Effects of cold temperature and water activity stress on the physiology of *Escherichia coli* in relation to carcasses

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STATEMENT OF CO-AUTHORSHIP

This thesis includes work, which has been published or submitted for publication in a peer-reviewed journal. More details for each chapter are described in the section of "Publications Arising from the Thesis". Proportionate co-author contributions were as follows:

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- Dr. Thea King from CSIRO Food and Nutritional Sciences (Australia) contributed to data analysis, interpretation of some results and development of the manuscripts.
- Dr. Kari Gobius from CSIRO Food and Nutritional Sciences (Australia) provided general guidance
- Assoc. Prof. John P. Bowman from Food Safety Centre, University of Tasmania (Australia) provided directional advice as supervisor, and contributed to the idea and manuscript preparation.

- Assoc. Prof. Tom Ross from Food Safety Centre, University of Tasmania (Australia) provided directional advice as supervisor, and contributed to the experimental design, implementation of the research program and manuscript preparation.

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) has emerged as an important food-borne pathogen of considerable public health concern. The majority of food-borne illnesses caused by EHEC, particularly serotype O157:H7, appear to be associated with undercooked meat and meat products. Although several intervention strategies are already in use to control carcass contamination, no single intervention is 100% effective in eliminating *E. coli* from carcasses. This indicates the need for developing an effective intervention. The Australian meat industry has a particular interest in evaluating the potential effect of combined cold and osmotic stresses on *E. coli*, as occurs during carcass chilling. To this end, the present thesis aims to provide a comprehensive understanding of the growth kinetics and cellular responses of *E. coli* O157:H7 strain Sakai subjected to conditions relevant to low temperature and water activity conditions experienced during meat carcass chilling in Australia where cold air chilling, rather than spray chilling, is routinely employed and leads to a temporary reduction in temperature as well as reduction in water activity during chilling. Despite that those temperature and water activity conditions are not lethal to *E. coli*, other studies have reported a decline in *E. coli* viability during cold air carcass chilling that could be exploited to deliberately reduce the prevalence of *E. coli* on meat.

An initial study employed an integrated transcriptomic and 'shotgun' proteomic approach (using cDNA microarray and 2D-LC/MS/MS analysis, respectively), to characterize the genome and proteome profiles of *E. coli* under steady-state conditions of cold temperature and water activity stress. Expression profiles of *E. coli* during exponential growth at 25°C a_w 0.985, 14°C a_w 0.985,

25°C a_w 0.967, and 14°C a_w 0.967 was compared to that of a reference culture (35°C a_w 0.993). Gene expression and protein abundance profiles of *E. coli* were more strongly affected by low water activity (a_w 0.967) than by low temperature (14°C). Predefined group enrichment analysis revealed that the universal response of *E. coli* to all low temperature and/or low water activity conditions included activation of the master stress response regulator RpoS and the Rcs phosphorelay system involved in the biosynthesis of the exopolysacharide colanic acid, as well as down-regulation of elements involved in chemotaxis and motility. Characterizing the physiology of *E. coli* under chilling and water activity stresses provided a baseline of knowledge to better interpret, and potentially exploit, this pathogen's responses to dynamic environmental conditions that occur during carcass chilling.

To gain deeper insight into the potential mechanisms controlling population responses of *E. coli* under dynamic low temperature and water activity conditions, a series of studies were undertaken to investigate the growth kinetics and time-dependent changes in its global expression upon a sudden downshift in temperature or water activity. Growth response of *E. coli* to a temperature downshift below 25°C was assessed. All downshifts induced a lag phase of growth before cells resumed growth at a rate typical for the temperature experienced. By contrast, shifting *E. coli* to low water activity (below a_w 0.985) caused an apparent loss, then recovery, of culturability. Exponential growth then resumed at a rate under characteristic for the water activity imposed. In transcriptomic and proteomic studies, *E. coli* responded to cold shock (from 35°C to 14°C) and hyperosmotic shock (from a_w 0.993 to a_w 0.967) by changing expression of genes and proteins involved in several functional groups and metabolic pathways. A number of these changes were found to be common for both cold and osmotic stresses,

although stress-specific responses were also observed. The adaptive strategies adopted by cells generally included accumulation of compatible solutes and modification of the cell envelope composition. Growth at cold temperature and low water activity, however, appeared to up-regulate additional elements, which are involved in the biosynthesis of specific amino acids. The present findings also highlighted the robust ability of *E. coli* to activate multiple stress responses by transiently inducing the activity of RpoE regulon to repair protein misfolding and aid the proper folding of newly synthesized proteins in cell envelope, while simultaneously activating the master stress regulator RpoS to mediate long-term adaptation under the stress conditions of low temperature and water activity.

A further study was carried out to examine the growth kinetics and molecular response of *E. coli* subjected to simultaneous abrupt downshifts in temperature and water activity (i.e. from 35°C a_w 0.993 to 14°C a_w 0.967). Exposure of *E. coli* to combined cold and osmotic shifts resulted in a complex pattern of population changes. Based on enumeration, this repeatable growth behaviour could be divided into three successive phases: (i) an initial decline in bacterial numbers followed by a period in which numbers increased rapidly; (ii) a second decrease in culturable cells and subsequent 'exponential-like' growth; and (iii) a constant population level, similar to the starting density before imposition of the combined shocks (i.e. analogous to a 'lag' phase), until 'true exponential' growth was (re)-established. From the proteomic analysis, it was found that the response of *E. coli* to downshifts in temperature and water activity involved a highly complex regulatory network, including changes in abundance of different groups of proteins. This regulatory network was mediated through a transient induction of the RpoE-controlled envelope stress response and activation of the master stress regulator RpoS and the Rcs system-controlled

colanic acid biosynthesis. Increase in abundance of several proteins with diverse functions was also observed, including those involved in the DNA repair system, the degradation of proteins and peptides, the amino acid biosynthetic pathways and the major processes of carbohydrate catabolism and energy generation.

Overall, the results presented in this thesis, and the interpretations based on them, provide a detailed understanding of the physiological responses of *E. coli* to dynamic changes experienced during carcass chilling under Australian conditions. Such knowledge will aid the development of more targeted, and less invasive approaches, for the meat industry to eliminate or control this pathogen.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

2D-LC/MS/MS	Two-dimensional liquid chromatography tandem mass spectrometry
AT	Adaptation time
a_w	Water activity
BHI	Brain-heart infusion
CFU	Colony forming unit
EHEC	Enterohemorrhagic *Escherichia coli*
ESI	Electrospray ionization
FRT	FLP recognition target
GT	Generation time
HCA	Hierarchical clustering analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
LT	Lag time
L	Length
MS/MS	Tandem mass spectrometry
MudPIT	Multidimensional protein identification technology
NCBI	National Center for Biotechnology Information
NSAF	Normalized spectral abundance factor
OD	Optical density
PCA	Principal-component analysis
RLT	Relative lag time

RT	Room temperature
SCX	Strong cation exchange
SpC	Spectral count
SpI	Spectral index
VBNC	Viable but non-culturable

PUBLICATIONS ARISING FROM THE THESIS

Chapter 2 Kocharunchitt, C., King, T., Gobius, K., Bowman, J.P., and Ross, T. (2011) Integrated transcriptomic and proteomic analysis of the physiological response of *Escherichia coli* O157:H7 Sakai to steady-state conditions of cold and water activity stress, *Mol. Cell. Proteomics*, doi:10.1074/mcp.M1111.009019.

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Chapter 4 Kocharunchitt, C., King, T., Gobius, K., Bowman, J.P., and Ross, T. Global genomic and proteomic responses of *Escherichia coli* O157:H7 Sakai during dynamic changes in growth kinetics induced by an abrupt downshift in water activity (manuscript under review for publication in the journal *Appl. Environ. Microbiol.*).

Chapter 5 Kocharunchitt, C., Bowman, J.P., and Ross, T. Proteomic response of *Escherichia coli* O157:H7 Sakai during dynamic changes in growth kinetics induced by simultaneous abrupt downshifts in temperature and water activity (manuscript under preparation).

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Since Enterohemorrhagic *Escherichia coli* (EHEC) was identified as a food-borne pathogen in an outbreak associated with undercooked ground beef in 1982 (Riley et al. 1983), it has emerged as a serious concern to public health. Many serotypes of EHEC are capable of causing human illnesses, which typically range from mild diarrhoea to the life-threatening hemolytic uremic syndrome (HUS) (McClure 2000; Cagney et al. 2004; Hussein and Bollinger 2005). Infection with EHEC can be acquired through direct transmission from person to person, from infected animals to people, or via water, but the major route reported has been through consumption of contaminated foods (Elder et al. 2000; McClure 2000; Vanselow et al. 2005).

EHEC, particularly serotype O157:H7 is considered a significant concern to the meat industry. Consumption of foods of bovine origin has been most frequently implicated in the outbreaks and sporadic cases caused by this pathogen. Numerous studies have also identified ruminant animals as the principle reservoir of EHEC with the pathogen occurring in the faeces, rumen contents, hide and derived carcasses (Elder et al. 2000; McClure 2000; Cagney et al. 2004). As a consequence, the meat industry has adopted several intervention strategies to control or reduce microbial contamination from the carcasses throughout the red meat supply chain. Such interventions including knife trimming, hot water washes, organic washes, steam vacuuming etc. have led to improvements in shelf life and safety of fresh meat. However, no single intervention can provide 100% assurance of safe, wholesome meat (Midgley and Small 2006). This clearly

indicates the need for developing additional or improving existing interventions that are more effective in eliminating or controlling the pathogens on the carcasses, and do not have negative impacts on meat quality.

Conventional air-chilling of carcasses has been implemented as an intervention in the meat industry in Australia and New Zealand. It involves the exposure of carcasses to a flow of refrigerated air. This consequently results in a dramatic decrease in temperature and, to a lesser extent, water activity (a_w) on the carcass surfaces, and thus has a significant impact on the growth and survival of bacteria, including *E. coli* (Gill and Bryant 1997; Anonymous 2004, 2007; Lenahan et al. 2009). However, bacteria are able to sense changes in their surrounding environment, and induce a response involving cellular defense mechanisms through a cascade of regulation of gene expression and protein activity (Chung et al. 2006). Such responses allow bacterial cells to survive under more severe conditions, enhance their tolerance to other stresses (i.e. cross protection), and possibly to enhance their virulence (Chung et al. 2006; White-Ziegler et al. 2008). Furthermore, it has been reported that bacterial cells, in response to adverse conditions, may lead to transition to a survival state, namely the 'viable but non-culturable' (VBNC) state. In this state, they lose their culturability (i.e. the ability to produce colonies) on conventional growth media on which they would normally grow or develop into colonies under standard conditions, yet display metabolic activity and possibly retain their pathogenic capacity (Golovlev 2003; Asakura et al. 2007). These VBNC cells also have the potential to regain their culturability when conditions become favourable for growth. Therefore, a detailed understanding of the physiological responses of *E. coli* to dynamic changes in temperature and water activity

relevant to carcass chilling is necessary to develop an effective hurdle to inactivate or control the growth of *E. coli* during this process.

1.2 *ESCHERICHIA COLI*

Escherichia coli, originally known as *Bacterium coli*, was first described in 1885 by Dr. Theodor Escherich, a German paediatrician, and was from the faeces of a healthy infant (Escherich 1989). This organism belongs to the family Enterobacteriaceae, and is described as a Gram-negative, non-sporulating, facultative anaerobic rod (Hussein and Bollinger 2005; Echeverry 2007). *E. coli* constitutes part of the normal microbiota of the gastrointestinal tract of humans and other warm-blooded animals (Eklund 2005; Ulukanli and Cavli 2006). This existence of *E. coli* in the host's intestine provides a means of symbiosis: the host offers a nourishing environment for the bacterium, while the bacterium provides a beneficial function in the host by giving essential nutrients to the intestinal epithelium and by promoting healthy immune responses (Eklund 2005). In addition, the food industry has been using non-pathogenic strains of *E. coli* since 1890 as an indicator of enteric pathogens, such as *Salmonella* in both foods and water. The presence of *E. coli* in foods or water usually indicates direct or indirect pollution of faecal origin, although it is not always indicative of the presence of other bacteria such as *Salmonella*. However, monitoring the numbers of a mesophilic indicator during food processing can provide important food safety/food hygiene information when environmental conditions cannot be monitored (Buchanan and Doyle 1997; Bjergbaek and Roslev 2005).

While more than a thousand strains of *E. coli* have been identified so far, only some strains are pathogenic, and can cause several forms of gastrointestinal diseases in humans (Eklund 2005).

These pathogenic *E. coli*, also known as 'diarrhoeagenic *E. coli*' can be classified into six different groups, based on their virulence factors, mechanisms of pathogenicity, clinical syndromes, and distinct serology. These are enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), diffusely adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EaggEC), enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC) (WHO & FAO2002; Derlinden et al. 2008). Among these, the EHEC group has become the most concerning as a food-borne pathogen. It has a significant impact on the meat industry due to its ecophysiological characteristics, the severity of its infection and number of involved cases and outbreaks (Jay 2000; Echeverry 2007). This review, therefore, focuses on enterohemorrhagic *E. coli* and its relevance to meats.

1.2.1 Significance of enterohemorrhagic *E. coli*

EHEC, alternatively known as verotoxigenic *E. coli* (VTEC) and Shiga toxin-producing *E. coli* (STEC) is characterized by its ability to produce a verotoxin (VT). This toxin was subsequently known as Shiga-like toxin, and more recently Shiga toxin (Stx). This is because its structure and biological activity are very similar to the toxin produced by *Shigella dysenteriae* type 1 (Buchanan and Doyle 1997; Fairbrother and Nadeau 2006). The designation of EHEC strains is based on the serological identification of specific antigens (i.e. O and H types) that are produced by their structural components. The O antigens refer to somatic lipopolysaccharides, whereas the H antigens are components of the bacterial flagellum (Hussein and Bollinger 2005).

EHEC was first recognized in the United States in 1982 when two outbreaks of severe bloody diarrhoea were associated with consumption of undercooked hamburger patties in a chain of fast

food restaurants (Riley et al. 1983). Since then, several outbreaks and sporadic cases of EHEC infection have been reported globally, and appear to be linked to the consumption of foods of bovine origin, as well as various other foods such as fruits and vegetables (Elder et al. 2000; McClure 2000; Vanselow et al. 2005; Buchholz et al. 2011). The majority of these have involved O157 EHEC, particularly *E. coli* O157:H7 (Elder et al. 2000; McClure 2000; Vanselow et al. 2005). However, there has been an increasing number of human illnesses caused by other serotypes of EHEC (i.e. non-O157 EHEC), mainly O26, O103, O118 and O145 (WHO & FAO 2002). In Australia, only few cases of disease have been implicated with *E. coli* O157:H7. Outbreaks linked to O157:H$^-$, O111:H$^-$, O113:H21 and O26:H11 are predominant (Fegan and Desmarchelier 1999).

1.2.2 Human illnesses associated with enterohemorrhagic *E. coli*

EHEC infections can cause a variety of clinical symptoms, mainly among children and the elderly. These can range from asymptomatic infection to the life-threatening complications. The number of EHEC cells required to cause disease is also thought to be very low, estimated from 1-100 bacterial cells (Vanselow et al. 2005; Derlinden et al. 2008). Therefore, the slightest presence of EHEC on foods can lead to serious infection.

Typically, the illness begins with abdominal cramps and mild, nonbloody diarrhoea over the first 24-48 h. This may later develop into overtly bloody diarrhoea (i.e. hemorrhagic colitis, HC) accompanied by severe abdominal pain and moderate dehydration during the next 4-10 days (Buchanan and Doyle 1997; WHO & FAO 2002). It has also been reported that 70% or more of the infected patients will progress to HC (WHO & FAO 2002). Several life-threatening

complications of EHEC infection may occur, of which hemolytic uremic syndrome (HUS) is the most common. This complication, which occurs most often in children under the age of 10, arises approximately a week after onset of gastrointestinal symptoms and is characterized by pallor, intravascular destruction of red blood cells (microangiopathic haemolytic anemia), lowered platelet counts (thrombocytopenia), lack of urine formation (oligo-anuria), swelling (edema) and acute kidney failure. More than half of HUS patients require dialysis and the mortality rate is 3-5% (Buchanan and Doyle 1997; Hussein and Bollinger 2005). Another life-threatening complication, thrombotic thrombocytopenic purpura (TTP), which involves central nervous system abnormalities, may also occur. TTP in contrast to HUS is more common in adults than in children. Other illnesses of EHEC infection include stroke, diabetes mellitus and necrotizing colitis (Hussein and Bollinger 2005).

1.2.3 Growth parameters

The growth and survival of *E. coli* are dependent upon a number of intrinsic and extrinsic environmental parameters. Intrinsic parameters can be defined as properties that are inherent to the food itself such as pH, a_w and nutrient content, whereas extrinsic parameters are those related to the storage environments including temperature, relative humidity and gaseous atmosphere (Buchanan and Doyle 1997). For any environmental parameter, bacterial cells are able to grow only over a limited range, which can be characterized by upper and lower limit for growth as well as optimal level for growth rate (Jay 2000).

E. coli has a wide temperature range for growth, and is generally able to maintain balanced growth, from 8°C to 49°C, with an optimum of 37°C. Increasing the temperature above 40°C or

lowering it below 20°C results in progressively slower growth rate, until the growth stops at the maximum temperature of 49°C, or the minimum of 8°C (Jones et al. 1987; Jay 2000). It has been reported that EHEC strains respond to temperature in the same manner as non-EHEC strains, with the exception of serotype O157:H7. Doyle and Schoeni (1984) demonstrated that many of O157:H7 do not grow well in the temperature range from 44°C to 45.5°C. However, this finding is inconsistent to the study of Salter (1998), showing that *E. coli* O157:H7, except *E. coli* O157:H- could grow at temperatures above 45°C.

E. coli can grow at pH values varying from 4.6 to 9.5 (Jay 2000), with similar growth rates at pH between 5.5 and 7.5. However, the minimum pH for growth depends on the interaction of pH with other growth parameters such as temperature (Buchanan and Doyle 1997; Presser et al. 1998). The type of acid (i.e. organic *versus* inorganic) and acid concentration are also known to influence the effect of pH on *E. coli* growth. A study on the relative inhibitory activity of organic acids including citric, lactic, and acetic on *E. coli* O157:H7 had shown to be increasing, respectively (Abdul-Raouf et al. 1993). It has also been reported that they could survive virtually unchanged 2 h to 7 h after exposures at pH 2.5. Their acid resistance provides the ability to survive in acidic foods and may explain their ability to survive passage through the stomach and cause infection at low doses (Buchanan and Doyle 1997; WHO & FAO 2002). In addition, EHEC strains were originally thought to have a higher degree of acid tolerance than generic strains (Buchanan and Doyle 1997), although this has later been shown not to be the case (Duffy et al. 2000; King et al. 2010).

The a_w for *E. coli* growth appears to range from 0.950 to 0.999 with an optimum of 0.995 (Lake et al. 2002). A study on the growth kinetics of a three-strain mixture of *E. coli* O157:H7 showed that the pathogens have a minimum a_w for growth of 0.980 when subjected to three non-ionic solutes such as mannitol, sorbitol and sucrose (Buchanan and Bagi 1997). In addition, it has been reported that the minimum a_w for growth is temperature-dependent by increasing the tolerance to desiccation at refrigeration temperature (Buchanan and Doyle 1997).

1.2.4 Enterohemorrhagic *E. coli* and carcasses

EHEC, particularly serotype O157:H7 is a significant concern to the meat industry. As noted in Section 1.2.1, consumption of foods of bovine origin has been most frequently implicated in the outbreaks and sporadic cases caused by this pathogen. Epidemiological evidences coupled with studies of foods associated with these illnesses have also identified ruminant animals, especially cattle as the primary reservoir of EHEC (Elder et al. 2000; McClure 2000; Cagney et al. 2004). Carriage in animals generally is asymptomatic, in which EHEC resides in the gastrointestinal tract and can be shed in the faeces. Shedding appears to be seasonal with the peak during summer and early autumn. Prevalence surveys conducted on fed cattle have also found that the O157 EHC faecal shedding rates during these times of the year can be as high as 61% (Heuvelink et al. 1998; McClure 2000; Barkocy-Gallagher et al. 2003; Hussein and Sakuma 2005).

Microbial contamination occurs inevitably in the conversion of live animals to meat products (Mead 1994). Although the muscle tissue of red meat animals is sterile, meat carcass can be contaminated with microbes during processing, storage and handling (Anonymous 2007). It has

been reported that faecal contamination of the external surfaces of hides, hooves and hair of animals can result in the transfer of high levels of EHEC to the surfaces of carcasses, particularly during the process of hide removal (Bell 1997; Baird 2005; Doyle and Erickson 2006). EHEC can also contaminate the carcass during evisceration if EHEC in the gut of the animals makes contact with the carcass, and later during production, carcass may be contaminated with EHEC that originates from contaminated processing tools, such as meat grinders (Farrell et al. 1998).

1.2.5 Preventive strategies for enterohemorrhagic *E. coli* during carcass production

Because meat is an important element in the diet of most people, its safety must be assured by effective interventions at various stages of the production chain, literally from 'farm to fork' but most effectively post-slaughter (Hinton 2000). Many of these control measures are interventions that aim to prevent or reduce microbial contamination on carcasses and derived meat products. The interventions currently used in the meat industry include animal cleaning, chemical dehairing at slaughter, spot-cleaning of carcasses before evisceration by knife-trimming or steam and vacuum, spraying, rinsing, or deluging of carcasses before evisceration and/or before chilling with water or chemical solutions (for critical review see Sofos et al. 1999; Koohmaraie et al. 2005).

Of particular relevance to this project, air-chilling is widely used as an intervention in the meat industry in Australia and New Zealand. This process typically involves the exposure of carcasses to a flow of cold air, and is used successfully as a control point (CP) in the hazard analysis critical control point (HACCP) system (Anonymous 2004; Lenahan et al. 2009). All meat processors are now required to validate their carcass-chilling procedures to comply with the

Australian Meat Standard AS 4696:2007 (Anonymous 2007). In the context of microbiology, carcass air-chilling generally leads to a reduction of temperature and a_w (at least temporarily) at the surfaces of meat carcass. This consequently creates environmental conditions that are stressful and that have a significant impact on the growth and survival of microorganisms, including *E. coli* (Gill and Bryant 1997; Anonymous 2007; Lenahan et al. 2009). Previous studies have also demonstrated that conventional chilling showed promising results in reducing *E. coli* numbers by up to 2 log unit over 24-36 h (Bacon et al. 2000), despite that there is a report showing that the air chilling achieved only a slight reduction (0.3-0.7 log unit) in microbial populations on carcasses (Thomas et al. 1997). Therefore, the carcass-chilling process has a potential to be developed further to optimize its efficacy in reducing or controlling microbes on carcasses.

1.3 PHYSIOLOGICAL RESPONSES OF *E. COLI* TO LOW TEMPERATURE AND WATER ACTIVITY STRESS

Changes in environmental conditions can have a significant impact on the growth and survival of bacteria, including *E. coli*. To adapt to such environmental changes, bacteria have evolved the capability to induce a stress response, which involves a cascade of regulations in gene expression and protein activity for the purpose of their survival. This stress response enables bacterial cells to protect vital processes and restore cellular homeostasis, as well as enhancing resistance against other environmental stresses (i.e. cross protection) (Aertsen and Michiels 2004; Chung et al. 2006; Giuliodori et al. 2007).

1.3.1 Cold stress and adaptation

Variations in the surrounding temperature are one of the most common stresses for all living organisms. Bacteria, in response to significant shifts to either higher or lower temperatures in their natural habitats, are equipped with cellular mechanisms to respond and adapt to these changes in various ways (Cao-Hoang et al. 2008; Phadtare and Inouye 2008). Extensive research on heat shock response and adaptation have been undertaken as heat shock causes well-defined damage to cells, including protein unfolding or denaturation (Phadtare 2004). On the other hand, the effect of cold shock was initially thought to be of a more general nature, for example the slowing down of metabolic activities. However, recent observations have changed this outlook on cold shock response, and have found that cells respond to the cold shock or cold stress in a specific manner at various levels (Phadtare 2004; Phadtare and Inouye 2008).

A rapid transfer of an exponential culture of the mesophilic bacterium *E. coli* from a temperature within the normal physiological range (i.e. from approximately 20°C to 38°C) to temperatures below 20°C has induced a cold shock response (Golovlev 2003; Giuliodori et al. 2007). This means that *E. coli* cells present in soil and natural aquatic systems are under cold stress conditions most of the time (Golovlev 2003). Furthermore, it has been demonstrated that shifting *E. coli* culture from 37°C to 10°C resulted in a lag period of cell growth for approximately 4 h followed by growth with a generation time of 24 h (Jones et al. 1987). During this transient arrest of cell growth (also known as acclimation phase), many physiological changes occur, including considerable alteration in the physicochemical state of cell membranes, stabilization of the secondary structures of nucleic acids (i.e. deoxyribonucleic acid [DNA] and ribonucleic acid [RNA]), inefficient folding of some proteins, and translational block due to hampered ribosome

movement (Baneyx and Mujacic 2003; Golovlev 2003). Most notably during the acclimation phase, synthesis of a number of specific proteins, called 'cold shock proteins' (CSPs) is transiently induced that aid bacterial cells to resume their growth at low temperature. Production of these cold shock proteins reaches a maximum induction during acclimation phase, with the level generally dependent upon the magnitude of the temperature downshift (Jones and Inouye 1994; Ermolenko and Makhatadze 2002). Eventually, the synthesis of the proteins drops to a lower basal level, and a new steady-state level of protein synthesis and growth are established, which is lower than before the cold shock (Ermolenko and Makhatadze 2002; Phadtare 2004).

1.3.1.1 Membrane composition upon temperature downshift

Temperature is one of the important factors influencing the composition, organization and function of biological membranes. Bacterial cell membranes generally adjust their lipid composition in accordance to changes in environmental temperature (Ulusu and Tezcan 2001). Under normal conditions, the membranes are in a liquid crystalline form, whereas the membranes upon temperature downshift, undergo a reversible transition to a gel phase (i.e. decrease in fluidity), affecting the membrane-associated functions such as active transport and protein secretion (Yamanaka 1999; Phadtare and Inouye 2008). The bacterial cells have developed various mechanisms to compensate for this transition in order to maintain the flexibility of their membranes. One of these is to change the degree of saturation of membrane phospholipids by increasing the proportion of unsaturated fatty acids (UFAs) in the membrane lipid composition. Others include shortening the fatty acid chain length and altering fatty acid branching from *iso*- to *anteiso*-branching (Chung et al. 2006; Phadtare and Inouye 2008).

E. coli respond to low temperature by increasing the proportion of *cis*-vaccenic acid (C18:1) at the expense of palmitic acid (C16:0) to adjust their fatty acid composition (Yamanaka 1999; Chung et al. 2006). It has been found that *E. coli* cells produced *cis*-vaccenate at 30 s after a temperature shift from 42°C to 24°C (Thieringer et al. 1998). This conversion is catalyzed by β-ketoacyl-ACP (acyl carrier protein) synthase II, encoded by *fabF*, which is considered to play a key role in fatty acid composition. As a result, the membrane lipid composition is modified to have an increased proportion of unsaturated fatty acids, and consequently, diunsaturated phospholipids, which have a lower melting point and a greater degree of flexibility compared to saturated phospholipids. This type of response is known as 'homeoviscous adaptation' (Yamanaka 1999; Phadtare and Inouye 2008). It is also worthwhile noting that the synthesis of β-ketoacyl-ACP synthase II is not cold inducible, but its activity is induced at low temperature (Yamanaka 1999). However, a study on membrane lipid composition upon cold shock has shown that with alteration in the medium composition and environmental conditions, *E. coli* was able to grow at 10°C without changing the degree of saturation and the isomeric composition of their fatty acids, as at 37°C. Therefore, changes in the fatty acid composition of cells may not be considered to be a typical response of *E.* coli upon cold shock (Jones and Inouye 1994; Golovlev 2003).

1.3.1.2 Synthesis of cold shock proteins

In response to cold shock, *E.* coli cells transiently induce synthesis of at least 17 different cold shock proteins, which have been mainly identified by two-dimensional gel electrophoresis (Jones and Inouye 1994; Jiang et al. 1997; Baneyx and Mujacic 2003; Chung et al. 2006). These proteins are essential for the cells to resume growth at the low temperature, and have been shown

to function in various cellular processes such as transcription, translation, mRNA degradation, protein synthesis and recombination (Thieringer et al. 1998; Chung et al. 2006). These cold shock proteins have also been found to share a high degree of similarity with DNA homology in food-borne spoilage, and pathogenic Gram-positive and Gram-negative bacteria (Yamanaka et al. 1998; Chung et al. 2006).

The cold shock proteins can be conventionally categorized into two classes, based on their degree of induction by low temperature as described by Thieringer et al. (1998) (Fig. 1.1). Class I cold shock proteins are expressed at an extremely low level at 37°C, but undergo more than a 10-fold induction in response to temperature downshift. Class I includes CspA, CspB CspG, CspI, CsdA, ribosome-binding factor A (RbfA), NusA, and polynucleotide phosphorylase (PNPase). In contrast, class II proteins are easily detectable at certain levels at 37°C, but are induced moderately (2-10 fold increase) upon cold shock. These are initiation factor-2 (IF-2), DNA-binding protein H-NS, RecA, α-subunit of DNA gyrase (GyrA), Hsc66, HscB, trigger factor (TF), dihydrolipoamide acetyltransferase and pyruvate dehydrogenase (lipoamide) (Thieringer et al. 1998; Yamanaka et al. 1998; Baneyx and Mujacic 2003).

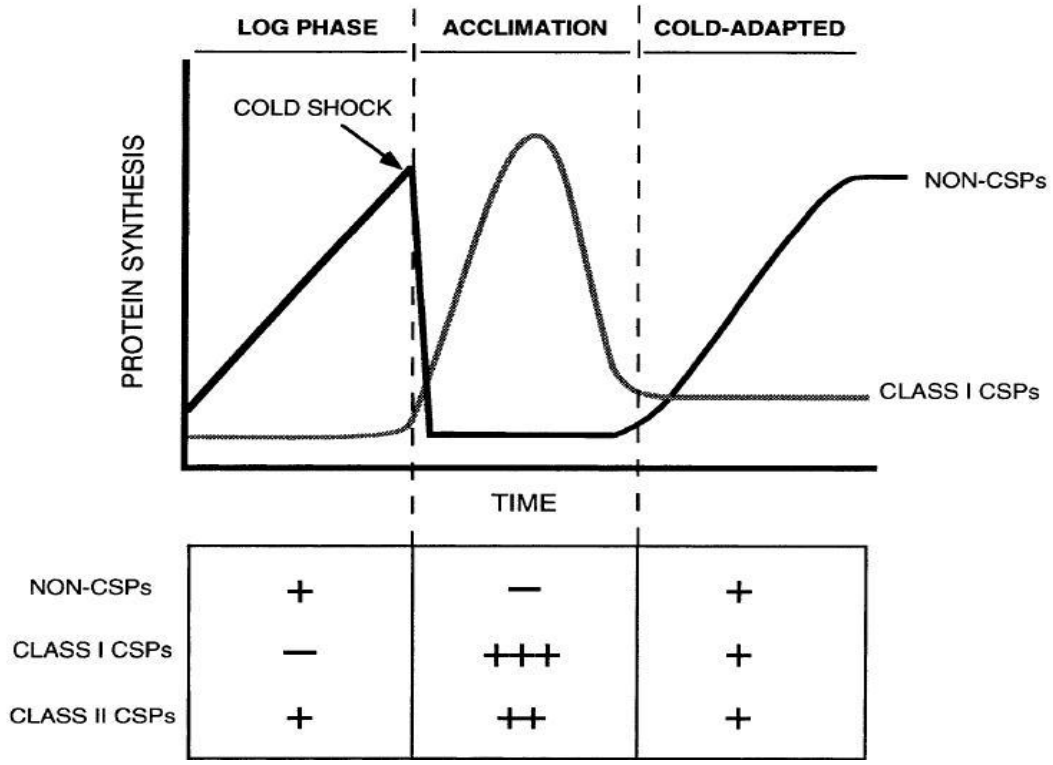

Fig. 1.1. Schematic representation of the synthesis of cold shock proteins (CSPs) in *E. coli* in response to the cold shock stress. During the acclimation phase, the synthesis of non-cold shock proteins is suppressed, whereas cold shock proteins, particularly class I cold shock proteins, are dramatically induced. After cells become cold adapted, the synthesis of class I cold shock proteins is subsequently repressed and non-cold shock protein synthesis is resumed (Thieringer et al. 1998)**.**

The CspA family

Among the cold shock proteins in *E. coli*, there is a family of structurally related proteins, named the CspA family, consisting of nine members (i.e. CspA to CspI). Each member has small molecular weight of approximately 7 kDa and shares between 29% and 83% identity with one another. The members of the family CspA have been shown to have an essential role during cold shock adaptation, although some also have important functions under normal growth conditions (Yamanaka 1999; Ermolenko and Makhatadze 2002).

The major cold shock protein, CspA

CspA, originally known as F10.6 and CS7.4 is the first protein described as a major cold-shock protein in *E. coli*. This protein is virtually undetectable at 37°C, but its production may reach 13% of the total cellular protein during the first hour after a temperature downshift from 37°C to 15°C (Jiang et al. 1997; Thieringer et al. 1998; Yamanaka 1999). CspA appears to bind cooperatively to RNA and single-stranded DNA (ssDNA) without apparent sequence specificity and with low binding affinity ($K_d \sim 3 \times 10^{-5}$ M). Its binding destabilizes the secondary structure in mRNA molecules, which renders mRNA more susceptible to RNase degradation. The low binding affinity of CspA to RNA also helps to facilitate ribosome movement, which would not be hampered by the CspA binding. Therefore, CspA is speculated to play the role of RNA chaperone, which is involved in the transcriptional regulation of CspA itself, and at least two other cold shock genes, *gyrA*, encoding DNA gyrase α-subunit, and *hns*, encoding H-NS (Yamanaka et al. 1998; Yamanaka 1999; Chung et al. 2006). It also may be involved in chromosome condensation and the stabilization of various kinds of RNA at low temperature (Golovlev 2003).

The expression of *cspA* gene has been reported to be regulated at the levels of transcription, mRNA stability (i.e. post-transcription) and translation. The *cspA* gene located at 80.1 min on the *E. coli* chromosome is monocistronically transcribed in a clockwise direction (Yamanaka 1999). The gene has a strong promoter, which is equipped with two unique motifs (Fig. 1.2). The first motif is the presence of an AT-rich sequence immediately upstream of the -35 region, which functions as the UP element and is directly recognized by the α-subunit of RNA polymerase, while another motif is a TG motif immediately upstream of the -10 region, which together is

called the extended -10 region. These two motifs increase the strength of the *cspA* promoter by facilitating transcription initiation, and hence conferring a high transcription activity of the *cspA* gene at both low and high temperature. It is also important to note that unlike heat shock genes, *cspA* does not require any *de novo* protein synthesis or specific sigma factors for transcription upon cold shock (Thieringer et al. 1998; Yamanaka et al. 1998; Yamanaka 1999). Indeed, *cspA* expression is still cold shock inducible, even when the *cspA* promoter is replaced with the *Ipp* promoter, a constitutive, strong promoter of a major outer membrane protein (Fang et al. 1997).

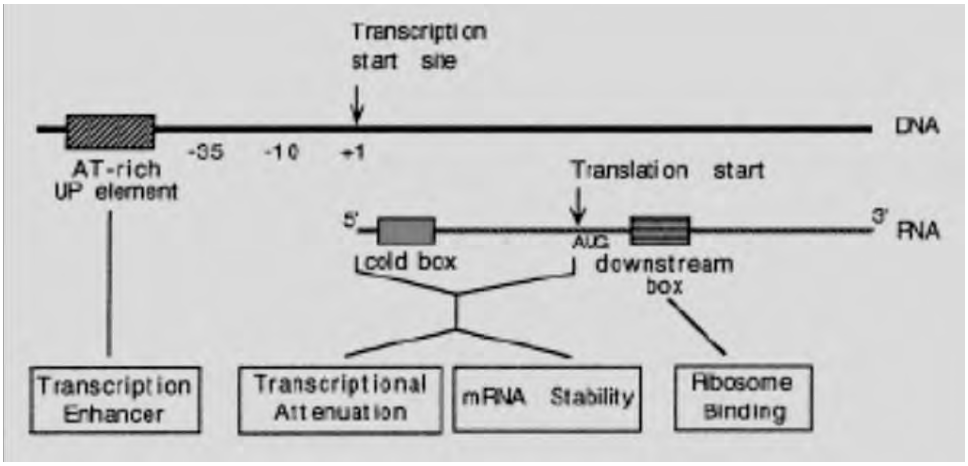

Fig. 1.2. Regulatory elements implicated in the cold shock induction of both *cspA* DNA and mRNA (Thieringer et al. 1998).

With the presence of the strong *cspA* promoter, large amounts of *cspA* transcripts are synthesized at both 37°C and 15°C. However, CspA is hardly detected at 37°C, because the *cspA* mRNA is extremely unstable, possessing a half-life of less than 12 s. By contrast, the *cspA* mRNA in response to the cold shock at 15°C becomes transiently stabilized with a half-life of more than 20 min, and subsequently loses its stability again, once cells are adapted to the cold shock (Fang et al. 1997; Yamanaka et al. 1998). The stability of the *cspA* mRNA has been suggested to be due

to the presence of an unusually long 5' untranslated region (5'-UTR) that contains a putative RNase cleavage site immediately upstream of the Shine-Dalgarno (SD) sequence, since the three-base substitution mutation around this region resulted in stabilization of the mRNA, allowing a constitutive production of CspA at 37°C. This also indicates that although the *cspA* promoter is active at 37°C, its transcript cannot be translated because of its extreme instability (Fang et al. 1997). The exact mechanism of the *cspA* mRNA stabilization upon cold shock is not yet clear, although a number of suggestions have been proposed. The enzyme RNase, which is responsible for the degradation of the *cspA* mRNA, may be somehow inactivated upon cold shock. Alternatively, the secondary structure of the *cspA* mRNA at 37°C may be highly susceptible to RNase and not be accessible to ribosome (Yamanaka 1999). In addition, at the 5' end of the UTR, there is a highly conserved unique 11 base long element called the cold box, which may form a stable stem-loop structure (Fig. 1.2). The cold box has been hypothesized to be either a binding site for a repressor molecule or a transcriptional pausing site, which is involved in auto-regulation of *cspA* expression. Binding of the putative repressor (possibly CspA) to the cold box during the acclimation phase interferes with transcription, or destabilizes the mRNA, leading to a suppression of CspA synthesis. Taken together, this has led to another suggestion, proposing that the putative cold box pausing site is somehow bypassed by RNA polymerase immediately after temperature downshift. However, once CspA reaches a threshold concentration, it binds to its own mRNA, thereby destabilizing the RNA polymerase elongation complex and attenuating transcription (Yamanaka 1999; Baneyx and Mujacic 2003).

Another important feature of the *cspA* mRNA for its translation upon temperature downshift is the presence of a 14 base downstream box (DB) located 12 bp after the *cspA* initiation codon

(Fig. 1.2). This DB sequence has been suggested to make the *cspA* mRNA more accessible to the cold-modified translation machinery and serves as an independent translational signal besides the SD sequence. The DB sequence is also complementary to the region near to the decoding region of 16S rRNA, called the anti-DB sequence of the 16S rRNA. The formation of a duplex between the DB sequence of mRNA and the anti-DB sequence of 16S rRNA was initially thought to enhance *cspA* translation initiation following cold shock, however this view is disputed and the exact mechanism of the enhancing effect on translation initiation by the DB remains unclear at present (Yamanaka 1999; Baneyx and Mujacic 2003; Phadtare 2004). Furthermore, the presence of the DB sequence is likely to help the *cspA* mRNA to bypass the requirement of cold shock ribosomal factors (i.e. CsdA, IF-2 and RbfA) during translation at low temperature. Taken together, the DB sequence may play an essential role to enhance mRNA translation during cold shock response, when the translation initiation for non-cold shock proteins is inhibited (Yamanaka et al. 1998; Yamanaka 1999). It is also very interesting to note that truncated *cspA* mRNA is still translatable, and when it is over-expressed at low temperature, cell growth is completely blocked. This effect, termed 'low-temperature antibiotic effect of truncated *cspA* expression' (LACE), is thought to be caused by the truncated *cspA* mRNA, which traps almost all ribosome before they become adapted to low temperature This is because the truncated mRNA still possesses the DB sequence that confer a very high capacity to form the initiation complex with non-adapted ribosome at low temperature. As a consequence, truncated CspA products are only produced, resulting in incapacity to repress the transcription of the truncated *cspA* gene (Thieringer et al. 1998; Yamanaka et al. 1998; Yamanaka 1999).

The CspA family of homologous proteins

Apart from CspA, three cold shock proteins (i.e. CspB, CspG, and CspI) have been found to be inducible to various magnitudes during a temperature downshift. They are thought to have a similar structure with similar or at least partially overlapping functions to CspA as a RNA chaperone, based on the similarities of their primary amino acid sequences (Rath and Jawali 2006; Giuliodori et al. 2007). In comparison with CspA, CspB, CspG and CspI are induced within a much narrower low temperature range. While CspA induction occurs over the broadest temperature range (30°C to 10°C), CspI induction occurs over the narrowest and lowest range (15°C to 10°C). Both CspB and CspG inductions occur at temperature ranging from (20°C to 10°C) (Yamanaka 1999).

CspC and CspE are constitutively expressed at both high and low temperature. They are implicated in chromosome condensation and cell division. Both have also been reported to be able to act as regulators of the production of a number of RpoS-dependant proteins involved in the general stress response network of *E. coli*. These include OsmY (osmotic stress and stationary phase), Dps (osmotic, oxidative stress and stationary phase), ProP (osmotic stress) and KarG (oxidative stress). CspE and CspC also regulate production of universal protein A (UspA), which is a protein responding to various stresses (Rath and Jawali 2006; Phadtare and Inouye 2008). The abundance of CspD in *E. coli* is induced during stationary phase and upon nutrition starvation. It has been shown that CspD binds to single-stranded regions of the replication fork and blocks DNA regulations, leading to cell death. In addition, the cellular functions of other members (i.e. CspF and CspH) of the Csp family are not yet known (Ermolenko and Makhatadze 2002; Rath and Jawali 2006).

In terms of gene expression upon cold shock stress, *cspB*, *cspG*, and *cspI* are thought to be regulated in the same manner as *cspA* (Jiang et al. 1997; Yamanaka 1999; Chung et al. 2006). They have been revealed to share several important features (Fig. 1.3). First, all genes contain the UP element upstream of the -35 region as well as the extended -10 region in their promoter, which enhance their transcription at low temperature. Secondly, they have a very long 5'-UTR (159, 161, 156, and 145 bases for *cspA*, *cspB*, *cspG*, and *cspI*, respectively), which has been shown to play a major role in the cold shock induction in the case of *cspA*. The third feature is the presence of a cold box sequence at the 5'end of their 5'-UTRs that play a role in auto-regulation to repress their own gene expression at the end of the acclimation phase. Lastly, their mRNA is equipped with a DB sequence downstream of the initiation codon, which plays a crucial role in the mRNA translation during the acclimation phase (Yamanaka et al. 1998; Yamanaka 1999).

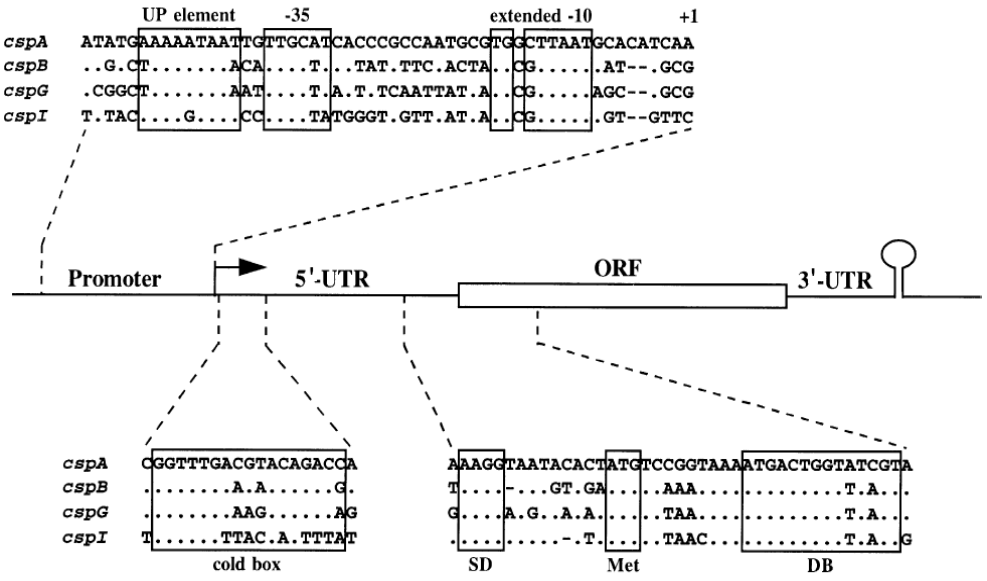

Fig. 1.3. Sequence of *cspA*, *cspB*, *cspG*, and *cspI* genes and their comparison (Yamanaka 1999).

Other cold shock proteins

In addition to the CspA family, other cold shock proteins have also been identified and shown to be essential for a variety of cellular processes upon temperature downshift. CsdA is a DEAD-box protein of helicases, which binds to the 70S ribosome. It appears to be involved in RNA-unwinding activity. RbfA is a ribosomal binding factor, which only interacts with 30S subunits. It has function as a late maturation or initiation factor, and is required for the efficient translation of most cellular mRNAs at low temperatures (Baneyx and Mujacic 2003; Phadtare 2004). NusA is involved in termination and antitermination of transcription, whereas PNPase is a ribonuclease that selectively degrades *cspA* mRNA at 15°C and repress production of CspA homologues at the end of acclimation phase (Phadtare 2004). RecA is involved in recombination and repair events in cells. IF-2 is a translation initiation factor that facilitates the initiation of tRNA (fMet-tRNA) binding to the 30S subunit. GyrA is the subunit of topoisomearse DNA gyrase. H-NS is a nucleoid-associated, DNA-binding protein. This histone-like protein has high affinity to bent DNA and probably promotes chromosome condensation at low temperature (Golovlev 2003; Phadtare 2004).

Trigger factor (TF) is a molecular chaperone functioning as a peptidyl-prolyl isomerase. It is induced, albeit at a modest level, after a lag period of 2-3 h upon cold shock, and its physiological role is thought to aid protein synthesis and folding (Yamanaka 1999; Phadtare 2004). A ribosome-associated protein of *E. coli*, pY is also induced by temperature downshift. It locates in the ribosomes of those cells that have reached the stationary phase of growth at a physiological temperature. It inhibits translation at the elongation stage by blocking binding of aminoacyl-tRNA to the ribosomal A site. In addition, both trehalose-6-phosphate synthase

(OtsA) and trehalose-6-phosphate phosphatase (OtsB), which are responsible for biosynthesis of trehalose, have been reported to be induced by cold shock in *E. coli* (Phadtare 2004).

Similar to those members of the CspA family in the class I cold shock proteins (i.e. CspA, CspB, CspG and CspI), mRNAs for CsdA, RbfA, NusA and PNPase contain the DB sequence in the coding region of their mRNA. They are therefore, efficiently translated without any requirement of ribosomal factors (Thieringer et al. 1998; Yamanaka 1999). By contrast, the regulation of other cold shock genes (i.e. class II cold shock genes) is not well understood at present. Expression of both *hns* and *gyrA* genes is regulated by CspA at the level of transcription upon temperature downshift. It is thought that CspA may help or stabilize the open complex formation for their transcription. Changes in DNA superhelicity is also likely to be involved in cold shock induction of these genes (Yamanaka 1999).

1.3.1.3 Trehalose accumulation

As described in more detail later (see Section "Neutral solutes"), trehalose is a non-reducing disaccharide present in many prokaryotic and eukaryotic organisms. Its accumulation has been reported to increase to very high levels (up to 500 mM) in bacterial and yeast cells, not only in response to heat shock and osmotic stress, but also upon exposure to low temperatures. Moreover, trehalose is thought to enhance cell viability when temperature is near freezing (Kandror et al. 2002; Phadtare 2004).

1.3.1.4 Chromosome dynamics

Various environmental factors such as changes in temperature or osmolarity can change the extent of DNA supercoiling, which is presumed to act as a thermosensor. Upon cold shock, cells condense chromosome by supercoiling and bending DNA, making the DNA more stable under this condition. Indeed, a transient increase in negative supercoiling of DNA has been observed after temperature downshift. This change is mainly found to affect the expression of various genes in such a way that the arrangement between the -10 and -35 regions of many promoters influences the recognition of some σ^{70} promoters by RNA polymerase (Phadtare 2004; Phadtare and Inouye 2008). The regulation of the DNA supercoiling appears to be essential for maintaining DNA transactions, such as replication, transcription and recombination. Notably, expression of DNA gyrase α-subunit (GyrA) that is induced upon cold shock may compensate for a decrease in the activity of DNA gyrase at low temperature, thereby providing the level of DNA supercoiling necessary for efficient DNA related functions. Two other cold shock proteins, RecA and H-NS are also thought to be involved in the reaction (Yamanaka 1999; Phadtare 2004).

1.3.1.5 Protein folding at low temperature

Protein misfolding was previously not considered as a major problem upon cold shock. However, recent reports have suggested that proper folding of proteins as well as refolding of cold damaged proteins are important following cold shock, although to a lesser degree than that upon heat shock (Yamanaka 1999; Phadtare and Inouye 2008).

Among cold shock proteins, trigger factor (TF) is known to catalyze the *cis*/*trans* isomerization of peptide bonds N-terminal to the proline residue. It appears to interact with nascent polypeptides on ribosomes, and influences the folding of newly-formed protein chains. TF can also bind with another chaperone such as GroEL to enhance its affinity for unfolded proteins. This presumably aids protein synthesis and folding, and may help to maintain pre-existing proteins in a functional form by promoting proper protein folding and refolding of cold shock-damaged proteins (Yamanaka 1999; Phadtare 2004). It has also been reported that artificially reduced TF content resulted in cell death faster, whereas cells with high levels of TF showed greater viability. This has led to the suggestion that TF is important for viability of cells at low temperature (Yamanaka 1999; Phadtare 2004).

1.3.1.6 Ribosome upon cold shock

Following a temperature downshift, cellular protein synthesis, especially at the translation initiation step becomes rate limiting for cell growth, resulting in an accumulation of 70, 50 and 30S ribosomes with a concomitant decrease in the number of polysomes (Jones and Inouye 1994; Thieringer et al. 1998). Previous studies on inhibition of the ribosome function either by cold-sensitive ribosomal mutations or by addition of translation inhibitors (e.g. chloramphenicol, tetracycline, spiramycin, fusidic acid, erythromycin etc.) have been shown to induce the cold shock response. Similarly, the addition of some translation inhibitors such as kanamycin also results in the induction of the heat shock response (Thieringer et al. 1998; Yamanaka 1999). These observations have led to the proposal that the state of the ribosome is another physiological sensor for induction of the thermal stress responses, and that the physiological

signal for the induction of the cold shock response is the inhibition of translation initiation upon the temperature downshift (Jones and Inouye 1994; Thieringer et al. 1998; Yamanaka 1999).

During the acclimation phase upon cold shock, protein synthesis is mainly blocked, most probably at the translation initiation step, indicating ribosome function is inhibited. While the synthesis of cold shock proteins is able to bypass this translational block, the synthesis of non-cold shock proteins requires some translational factors that are induced upon cold shock (Yamanaka 1999). These are ribosome-associated proteins, CsdA, RbfA and IF-2 (see Section "Other cold shock proteins"), which are important for efficient ribosomal function at low temperature. Based on the cold shock ribosome adaption model, all of these cold shock proteins appear to associate with the ribosomal subunits to convert the cold-sensitive, non-translatable ribosome into a cold-adapted translatable state upon cold shock. This, therefore, results in recovery of the synthesis of cellular protein, and cell growth (Thieringer et al. 1998; Yamanaka 1999).

Guanosine 5' diphosphate-3' diphosphate, (p)ppGpp is a signal or control molecule, which is involved with the stringent response in bacteria. Its level increases when there is a shortage of amino acids, causing the inhibition of tRNA and rRNA synthesis, and hence a decrease in translation. The level of (p)ppGpp is influenced by temperature changes (Jones and Inouye 1994). In *E. coli*, a temperature upshift results in an increase in the (p)ppGpp level, while a temperature downshift leads to a decrease of its level. Increased levels of (p)ppGpp have also been observed in bacterial cells under a nutritional stress. However, an abrupt downshift in temperature causes a decrease in translational capacity of the cell, triggering the decrease in the

(p)ppGpp level with the corresponding changes in gene expression under the cold shock response (Jones and Inouye 1994; Phadtare 2004). The decreased level of the (p)ppGpp also appears to positively affects the synthesis of the transcriptional and translational proteins as well as many cold shock proteins. Furthermore, the magnitude of the decrease in the (p)ppGpp level is proportional to the magnitude of the temperature downshift in which larger magnitude of the temperature shift leads to the larger decrease in its level. Therefore, the change in the (p)ppGpp level in response to the temperature downshift can be considered to be part of an adaptive response (Jones and Inouye 1994).

1.3.2 Osmotic stress and adaptation

Increasing osmotic pressure (lowering a_w) is among one of the most widely used methods to control the growth of food spoilage and pathogenic bacteria. It involves desiccation or addition of high amounts of osmotically active compounds (Beales 2004; Chung et al. 2006). Bacteria, including *E. coli*, have to maintain an internal pressure higher (or an internal a_w lower) than that in their environment. As a consequence, there is a tendency for water to flow into the cells, creating hydrostatic pressure exerted outwards on the cell wall. This pressure known as turgor pressure or turgor is essential for growth and division of the cells by providing the mechanical force for cell elongation (Csonka 1989; Chung et al. 2006). Therefore, the mechanisms that regulate the osmotic balance of organisms are central to the process of cell growth.

Hyper- or hypoosmotic shock leads to an instantaneous efflux or influx of water, which is accompanied by a concomitant decrease or increase in the cytoplasmic volume, respectively. Hypoosmotic shock generally results only in minor increases in cell volume, whereas

hyperosmotic shock causes considerable shrinkage of the cytoplasmic volume in a process called plasmolysis, and may lead to reduced respiration and growth arrest (Csonka 1989). The kinetics and extent of plasmolysis are dependent on the magnitude of the increase in the osmolarity of the medium but not on the nature of the solutes used, provided that the solutes are excluded from the cytoplasm by the membrane. If the osmotic shock is not too severe, plasmolysis is transient, and the cytoplasmic volume will increase due to osmotic adjustment made by the cells after adaptation involving uptake or synthesis of osmolytes (Csonka 1989).

1.3.2.1 Osmoregulation

E. coli responds both passively and actively to changes in the osmolarity of its surrounding environment (Wood 1999). Bacteria activate osmotic regulation or osmoregulation to cope with osmotic stress. A major role of osmoregulatory systems is to maintain turgor pressure within tolerable limits by adjusting the total osmotic solute pool of the cytosol (Csonka 1989). This is achieved initially by active uptake of charged solutes (e.g. potassium ions (K^+) and glutamate), followed by accumulation of compatible solutes either through *de novo* biosynthesis (e.g. trehalose) or through uptake from the external environment (e.g. glycine betaine and proline) (see Fig. 1.4 and 1.5 for summary) (O'Byrne and Booth 2002; Chung et al. 2006).

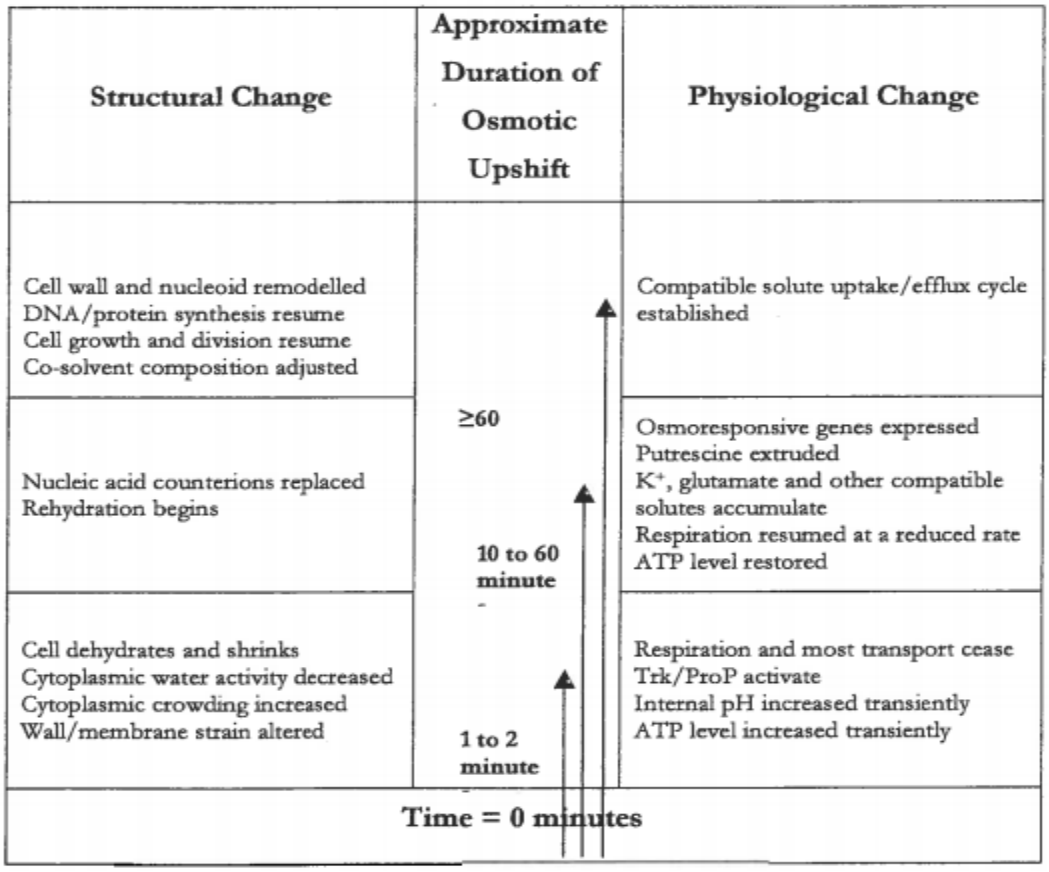

Fig. 1.4. Structural and physiological responses for *E. coli* to hyperosmotic shock. Osmotic upshift is imposed at time zero and proceeds in parallel along the indicated, approximate timescale. Reproduced from Wood (1999).

1.3.2.2 Compatible solutes

Accumulation of compatible solutes is a common response to osmotic shock. Compatible solutes are highly soluble, zwitterionic, pH neutral, and are usually end product metabolites. They generally do not interfere with biochemical processes (e.g. DNA replication, DNA-protein interactions, and the cellular metabolic machinery) even at high concentration. Many of them are also effective stabilizers of enzymes, providing protection not only against high salt but also against high temperature, freeze-thawing and drying (Yancey et al. 1982; Csonka 1989; Beales 2004). A variety of compatible solutes have been reported. These included K^+, amino acids (e.g.

glutamate, proline), amino acid derivatives (peptides, N-acetylated amino acids), quaternary amines (e.g. glycine betaine, carnitine), sugars (e.g. sucrose, trehalose), and tetrahydropyrimidines (e.g. ectoines) (Poolman and Glaasker 1998).

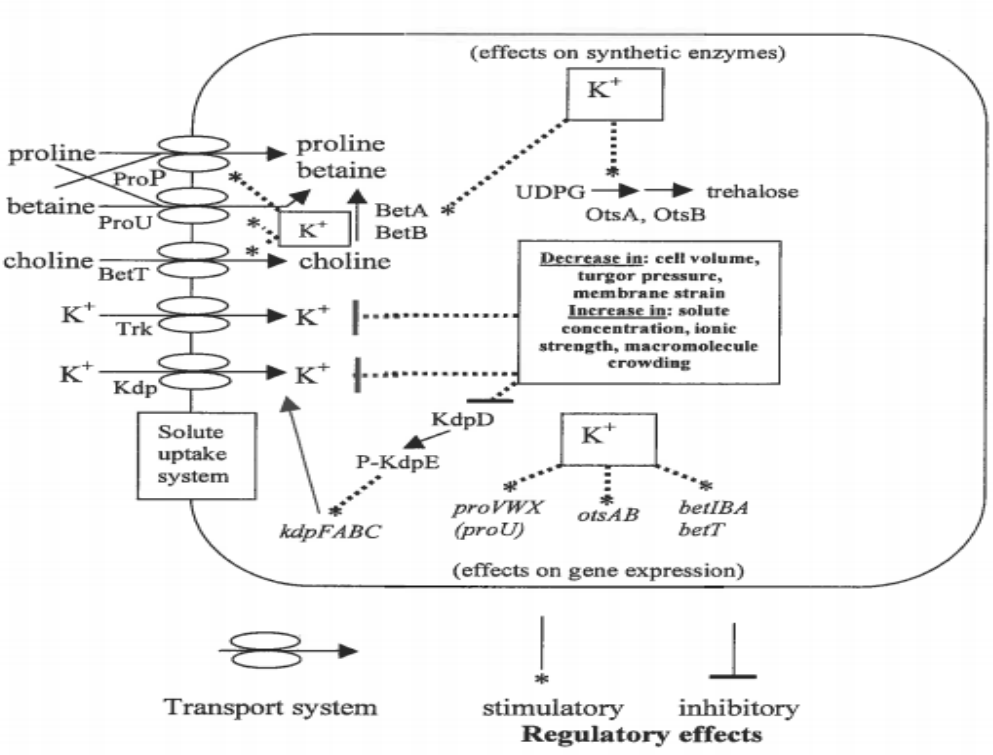

Fig. 1.5. Osmoregulatory system in *E. coli* during adaptation to high osmolarity. Changes in a_w are postulated to control the activity of transport systems, enzymatic steps, as well as the transcription of several genes. An increased concentration of cytoplasmic K^+ is believed to be the central regulatory signal, which triggers and coordinates other osmotic responses, although contradictory findings have also been reported. The major transport systems that accumulate compatible solutes are shown on the left. Regulation of biosynthetic events is shown at the top, and effects on gene expression are shown at the bottom. Solid arrows show movements of solutes, biochemical steps, or phosphorylation of KdpE by KdpD and transcription of the *kdpFABC* operon. The proposed regulators and their targets are connected by dashed lines. Reproduced from Csonka and Epstein (1996).

Not all compatible solutes are equal. Solutes, which are uncharged or zwitterionic, are generally more favourable to protein stability than ionic solutes. Following hyperosmotic shock, the immediate response of cells to restore intracellular osmotic pressure by accumulating K^+, glutamate and other ions can create a highly ionic cellular environment that is unfavorable for cell growth. This initial response is, therefore, only an interim solution (Record et al. 1998). Accordingly, a second phase of osmoregulation takes place, which involves accumulation of neutral compatible solutes (Poolman and Glaasker 1998; Record et al. 1998). The major neutral compatible solutes used by enteric bacteria are trehalose, proline, and zwitterionic quaternary ammonium compounds, of which betaine is most commonly encountered (Csonka 1989). In most eubacterial species, glycine betaine is the preferred compatible solute, and generally provides the highest level of osmotolerance, which reflects its favourable interaction with macromolecules (Poolman and Glaasker 1998).

Ionic solutes

Potassium ions (K^+)

Potassium ions are the most prevalent cations in the cytoplasm of bacteria. As a consequence, they serve as one of the major intracellular osmolytes that maintain turgor. The intracellular concentration of K^+ has been reported to be nearly proportional to the osmolarity of the growth medium. There is also a positive correlation between the intracellular content of this cation and the ability of bacteria to tolerate conditions of high osmolarity (Csonka 1989). Uptake of K^+ is shown to be the initial response of enteric bacteria to a hyperosmotic shock. Its accumulation is often accompanied by increases in the glutamate pool through *de novo* synthesis during growth at high osmolarity to maintain electroneutrality, although other processes such as accumulation

of other anions, putrescine excretion and proton efflux may contribute as well (Poolman and Glaasker 1998).

In *E. coli*, K^+ uptake is facilitated by two major transport systems (Trk and Kdp), which occurs via specific sites in the inner cell membrane. The Trk transport system is the predominant K^+ uptake system in *E. coli*, and is expressed constitutively with a modest affinity for K^+ (Csonka 1989). The uptake of K^+ by this system increases in response to osmotic upshift. The activity of Trk has been reported to be dependent upon the intracellular osmolarity at the time the external osmolarity is increased (Poolman and Glaasker 1998). In contrast, the Kdp transport system shows a relatively high affinity for K^+, and is not expressed under normal circumstances. Kdp scavenges K^+ when the external concentration of K^+ is low (Csonka 1989). Additionally, a minor transport system namely Kup has been reported. It is constitutive and is not influenced by medium osmolarity (Kempf and Bremer 1998).

Glutamate and other anions

In enteric bacteria, the most abundant osmotically regulated anion is glutamate (Measures 1975). Concomitantly with K^+ uptake, the synthesis of glutamate is strongly induced to counterbalance the accumulated positive charges. Accumulation of glutamate is thought to occur within a minute of hyperosmotic shock and is dependent on K^+ uptake. In *E. coli*, there are two pathways for glutamate synthesis, the *gltBDF*-encoded GS/GOGAT (glutamate synthetase) or the *gdhA*-encoded GDH (glutamate dehydrogenase) system. However, it has been reported that none of these systems is up-regulated after increased osmolarity, at least not directly (RoeBler and Muller 2001).

Apart from accumulation of glutamate, the levels of other anions have also been reported to increase substantially under high osmolarity. These include γ-glutamylglutamine, glutathione, and those of phosphorylated metabolites (e.g. dihydrozyacetone phosphate and 1,3-bisphosphoglycerate) (Csonka 1989; McLaggan et al. 1990). These changes are normally transient but last longer when cells are unable to accumulate glutamate (Csonka 1989).

Putrescine

Putrescine is the polyamine present in highest concentration in *E. coli*. It is a precursor of spermidine. Both putrescine and spermidine have been implicated in various aspects of *in vitro* RNA and protein synthesis (Munro et al. 1972). Although putrescine has been reported not to contribute much to cytoplasmic osmolarity (Csonka 1989), Munro et al. (1972) has found that exposure of the cells to hyperosmotic shock resulted in rapid excretion of putrescine. The same study also proposed that since putrescine is a divalent cation, each molecule of putrescine could be exchanged for two K^+ ions. Therefore, the osmotic strength of the cytoplasm could be increased without affecting the overall concentration of ions (Munro et al. 1972).

Neutral solutes

Trehalose

Under osmotic stress, accumulation of trehalose occurs via *de novo* synthesis as the predominant endogenous compatible solute in minimal medium or in the absence of exogenous compatible solutes such as glycine betaine and proline. The intracellular concentration of trehalose in *E. coli* usually accounts for up to 20% of the osmolar concentration of solutes (Csonka 1989; Kempf and Bremer 1998). Trehalose is synthesized by two enzymes encoded by the *otsAB*

(osmoregulated trehalose synthesis) operon. The *otsA* product, trehalose-6-phosphate synthase catalyzes the condensation of the precursors glucose 6-phosphate and UDP-glucose to form trehalose-6-phosphate. This intermediate is then used by the *otsB*-encoded trehalose-6-phosphate phosphatase to generate trehalose (Kempf and Bremer 1998; Purvis et al. 2005). The expression of the *otsAB* operon is induced by osmotic stress, which is entirely dependent on RpoS. The K^+-glutamate accumulated in osmotically stressed cells has also been reported to play a specific role for the trehalose synthesis pathway. The activity of the *otsA*-encoded trehalose-6-phosphate synthase appears to be strongly stimulated by K^+-glutamate and salts of other monovalent cations (Kempf and Bremer 1998).

While endogenously synthesized trehalose has an osmopretective function, exogenous trehalose is used as a carbon and energy source under high osmolarity growth conditions. At high osmolarity, the synthesis of a periplasmic trehalase encoded by *treA* (*osmA*) is induced. Its expression is partially dependent on RpoS. TreA hydrolyses extracellular trehalose to produce two glucose molecules. These glucose molecules are then taken up and converted to glucose 6-phosphate by the phosphotransferase system (Gutierrez et al. 1989; Kempf and Bremer 1998). The dual use of trehalose as a compatible solute and as a carbon source enables bacterial cells to grow on trehalose in media of high osmolarity, while maintaining high internal pools of trehalose. In addition, metabolism of endogenous trehalose is prevented by the repression of the *treBC* operon (Gutierrez et al. 1989).

Osmoprotectants

Osmoprotectants are defined as exogenously provided organic solutes that enhance the growth rate of bacteria in media of high osmolarity. They may themselves be compatible solutes, or act as precursor molecules that can be enzymatically converted into compatible solutes (Csonka 1989; Kempf and Bremer 1998). It has been reported that energy required to accumulate osmoprotectants may be less than that needed for the uptake of K^+ and the synthesis of glutamate. Therefore, the uptake of osmoprotectants (e.g. proline and glycine betaine) has priority over the synthesis of other compatible solutes (Csonka 1989; RoeBler and Muller 2001).

Two groups of transport systems, ProP and ProU are primarily responsible for the uptake of osmoprotectants across the cytoplasmic membrane. These transporters were originally identified as osmotically stimulated uptake systems for proline, but subsequent studies established their role in the uptake of a wide spectrum of osmoprotectants, of which betaine and proline are transported with high affinity (Csonka 1989; Kempf and Bremer 1998; Chung et al. 2006). Permeation of osmoprotectants across the *E. coli* outer membrane is achieved by passive diffusion through the non-specific porins OmpC and OmpF. Expression of the *ompC* and *ompF* structural genes is regulated in a reciprocal fashion by medium osmolarity. The synthesis of OmpC predominates in hypertonic media, whereas OmpF production occurs primarily under hypotonic conditions. Osmoregulation of these genes is mediated by the cytoplasmic membrane-embedded sensor kinase EnvZ and by the response regulator OmpR (Csonka 1989; Kempf and Bremer 1998).

The ProP transporter consists of a single integral membrane protein driven by cation symport. It mediates transport of a wide variety of osmoprotectants such as betaine, proline, and ectoine with similar affinities. Transport via ProP is greatly enhanced during osmotic upshift by a combination of 2- to 5-fold transcriptional induction and stimulation of the activity of the ProP protein (Csonka 1989; Kempf and Bremer 1998). Transcription of *proP* is directed from two promoters, P1 and P2, both of which are activated by osmotic stress. The activity of *proP*-P1 is repressed by the cAMP-CRP complex, whereas the activity of *proP*-P2 is regulated by RpoS and the nucleoid-associated protein Fis (Kempf and Bremer 1998). ProU is a binding protein-dependent transport system and a member of the family of ABC transporters. ProU exhibits a broad specificity for compatible solutes, but has a high activity and affinity for betaine. The ProU transport system contains three components, including the membrane-associated ATPase (ProV), the integral membrane protein ProW, and the periplasmic substrate-binding protein ProX. Transcription of the *proU* operon can be induced greater than 100-fold by high osmolarity, and induction can be detected within 4 min after hyperosmotic shock (Gowrishankar 1985; Csonka 1989; Kempf and Bremer 1998). Induction of the *proU* operon is not a direct response to an osmotic signal. This is because its induction appears to be delayed and reduced in extent when K^+ uptake is slow (Sutherland et al. 1986). The osmotically regulated promoter of the *proU* operon is recognized by the RpoD-RNA polymerase holoenzyme and there is a minor RpoS-dependent promoter located upstream of the major RpoD-dependent promoter (Csonka 1989). It is also postulated that expression of the *proU* operon is regulated by supercoiling, which is dependent upon the osmolarity of the medium (Kempf and Bremer 1998).

E. coli can synthesize glycine betaine by a two-step oxidation of the precursor choline with glycine betaine aldehyde as the intermediate. Extracellular choline is first taken up with high affinity by the proton-motive force-driven BetT transporter and with low affinity by the ProU system. It is then converted to glycine betaine through the sequential action of choline dehydrogenase and betaine aldehyde dehydrogenase, encoded by *betA* and *betB*, respectively (Csonka 1989; Kempf and Bremer 1998). All *bet* genes (i.e. *betTBA*) are typically regulated by betaine inhibitor, BetI and can be induced 7- to 10-fold by osmotic upshift. Expression of the *bet* genes is further enhanced by choline and is repressed by betaine (Csonka 1989).

1.3.2.3 Cell membrane compositions in response to osmotic stress

Apart from the accumulation of solutes in the cytoplasm, alterations in membrane lipid composition in response to low a_w have also been suggested to be part of the osmoregulation response in *E. coli* (Russell and Kougut 1985). In contrast to the effect of temperature on membrane composition, the major change in response to osmotic stress occurs in the head group of the lipids. Specifically, the levels of anionic phospholipids and/or glycolipids increase when external a_w is lowered by means of preservative solutes. This increase is to preserve the membrane lipids in the structure of the lipid bilayer, and allows for proper functioning of the membrane in the low a_w environment (McGarrity and Armstrong 1975).

1.4 VIABLE BUT NON-CULTURABLE STATE IN *E. COLI*

For many decades, culturability of bacteria has been used as a means to measure cell viability in which loss of culturability is assumed to indicate cell death (Nystrom 2003; Aertsen and Michiels 2004). However, this view was challenged by Xu et al. (1982) who first described the

'viable but non-culturable' (VBNC) state. When bacteria enter this state, they are apparently intact and metabolically active, but have lost the ability to undergo the sustained cellular divisions required to form a colony or grow on bacteriological media under standard conditions (Nystrom 2003; Oliver 2005; Keep et al. 2006).

While it is well known that non-differentiating bacteria are capable of developing starvation and stress responses through a series of differentiation-like genetic programs, the nature and biological significance of the VBNC state have been the topic of much interest and intense debate. Two main, but contrasting, theories have been proposed to explain this phenomenon (McDougald et al. 1998; Arana et al. 2007). One model proposes that the VBNC state may represent a survival strategy adopted by non-spore-forming bacteria when exposed to potentially adverse conditions. Such strategy is presumed to arise as a programmed physiological response involving the induction of global control networks to generate dormant survival forms, similar to spore formation (McDougald et al. 1998; Lleo et al. 2001; Arana et al. 2007). In this interpretation, the VBNC is the existence of viable cells in the state of survival, and these cells also should have the ability to reverse this program and become culturable again (i.e. a process of resuscitation) upon restoration to favourable environmental conditions (McDougald et al. 1998; Arana et al. 2007). On the contrary, the second model argues that bacterial non-culturability is due to cellular deterioration, and that the VBNC state may be a moribund condition in which cells become progressively debilitated until death is inevitable. These cells may maintain signs of metabolic activity for some time but are not able to resuscitate (McDougald et al. 1998; Boaretti et al. 2003). Interestingly, some studies have also suggested that the latter model is probably an adaptive strategy to ensure persistence of the bacterial population. Cells with reduced or no

colony-forming capacity may provide compounds such as energy nutrient substrates through cell lysis. These compounds then serve as a nutrient source for remaining culturable cells in the same population, and allow other cells to persist under the adverse conditions (Na et al. 2006; Arana et al. 2007).

There have been numerous reports of both Gram-positive and Gram-negative bacteria entering the VBNC state. These include bacteria of public concern like *Campylobacter* spp., pathogenic *E. coli* strains, *Listeria monocytogenes*, several strains of *Salmonella*, and *Vibrio vulnificus*, with the latter being the most extensively studied (Bates and Oliver 2004; Oliver 2005; Sardessai 2005). The conditions that have been identified to affect culturability vary between organisms. The stresses of limited nutrient availability and low temperature appear to be the main factors to induce the VBNC state, although variations in pH, osmotic stress, UV exposure and exposure to antimicrobial agents have also been reported (Bjergbaek and Roslev 2005; Na et al. 2006; Asakura et al. 2007). Furthermore, decontamination processes such as pasteurization of milk and chlorination of wastewater, which are normally assumed to be effective against bacteria may instead cause the cells to enter the VBNC state and survive in the environments (Oliver 2005; Sardessai 2005). Therefore, bacteria in the VBNC state may not be detected by standard microbiological testing methods, posing a potential threat to public health (Oliver 2005; Arana et al. 2007). Although there is no firm evidence that public health is at risk from the VBNC bacteria (Bogosian and Bourneuf 2001), understanding how bacteria enter into and exit from the VBNC state still has potential implications for detection and therapy (Keep et al. 2006).

1.4.1 Characteristics of the VBNC cells

In the VBNC state, bacterial cells usually reduce in size. Rod-like bacteria have been reported to change their shape to a coccoid (or very short rod) morphology (Yamamoto 2000; Oliver 2005). A number of major metabolic changes are also thought to occur. These include dramatic reductions in nutrient transport, respiration rates and macromolecular synthesis. Protein composition, ribosomal content, and possibly DNA arrangement have also been reported for most bacterial species in the VBNC state (Lleo et al. 2001; Oliver 2005; Arana et al. 2007). Furthermore, cytoplasmic membrane, fatty acid composition and cell wall peptidoglycan appear to be extensively modified. These modifications are believed to stabilize the cell wall and membrane, which may be essential for entry into the VBNC state (McDougald et al. 1998; Day and Oliver 2004). Indeed, a study on *Micrococcus luteus* in the VBNC state demonstrated that total lipid content decreased rapidly with the cytoplasm becoming denser in the non-culturable cells. These cells also maintained their morphological integrity, including an intact nucleoid and structured cytoplasm. However, their cell wall became thicker, and exhibited a loss of permeability by the cytoplasmic membrane. This loss of permeability may be one of the reasons for loss of culturability (Mukamolova et al. 1995).

In at least some species, the VBNC cells differ from those in the starvation survival state stimulated by nutrient deprivation or exhaustion, where the starvation response allows bacterial long-term persistence in a non-growing but culturable state. It has been reported that cells in both states represent distinct metabolic responses to confront adverse environmental conditions, although cells in both states undergo dramatic decreases in metabolic activity (Heim et al. 2002; Oliver 2005). Physiological differences of the two states can be identified such as by proteome

analysis (Heim et al. 2002) and cytological assay (e.g. changes in cell wall) (Signoretto et al. 2000). Such diagnostics have also been suggested to give a reliable determination for the VBNC state of cells (Keep et al. 2006). In addition, VBNC cells have been shown differ from sub-lethal cell injury and cell stress in which recovery of culturability is not the result of simply removing the VBNC-inducing signal (Grey and Steck 2001).

A number of methods have been employed to examine the viability of non-culturable cells. However, there is no single method that is suitable in all cases. The viability of bacteria can be assessed in populations (bulk assay) or in single cells (cytological assay) (Kell et al. 1998; McDougald et al. 1998). Bulk assays generally rely on detection of cellular metabolic activity (i.e. measurement of respiration rates). These assays require the bacterial population to be completely non-culturable otherwise it can be difficult to provide a correlation between biochemical activities and culturability (Kell et al. 1998; McDougald et al. 1998; Grey and Steck 2001). Although some studies have used bulk assays successfully (Rollins and Colwell 1986), cytological assays appear be preferred in many studies. This is because the cytological assays are based on growth-independent viability assays, such as labelling techniques that assess cell viability by the maintenance of stable cellular structure. These methods include the use of nucleic acid stains, redox indicators, membrane potential probes, which can be examined by microscopy or flow cytometry (Kell et al. 1998; Bogosian and Bourneuf 2001).

1.4.2 Resuscitation of VBNC cells

To prove the existence of the VBNC phenomenon as a physiological survival mechanism, it ultimately requires demonstrating the recovery of the culturable state from a population of non-

culturable cells (Bogosian and Bourneuf 2001; Bates and Oliver 2004; Oliver 2005). Such a reversal in physiology termed 'resuscitation' has been demonstrated in several species, and is thought to be genetically controlled because addition of translational or transcriptional inhibitors can prevent the resuscitation process (Oliver 2005). The resuscitation process is often triggered simply by the removal of the stress, which initially induced the VBNC response (Bogosian and Bourneuf 2001; Bates and Oliver 2004; Oliver 2005). There have also been numerous reports of resuscitation induced by other mechanisms such as nutrient addition, transfer to fresh medium and heat shock. However, most of these resuscitation processes were successful only with cultures, which had been in the VBNC state for only a short period of time (McDougald et al. 1998; Bogosian and Bourneuf 2001).

Yamamoto (2000) has proposed that resuscitation can be an extremely intricate and complex process, and may require the presence of special factors. These resuscitation factors have a biphasic role, which can induce entry to and exit from the VBNC state. They can be classified into three, non-exclusive categories. The first group of factors refers to the removal of the environmental stress. This could be done by adding nutrients to starved cells, changing to a suitable temperature or returning to the host organism in the case of obligate parasites (Yamamoto 2000). In many strains of *Vibrio*, the VBNC state is typically induced by a temperature downshift, and resuscitation from this state has been observed upon a temperature upshift (Micunoe et al. 2000). However, it is worthwhile noting that addition of nutrients (alone) may not be able to recover the culturable state of the VBNC cells (Whitesides and Oliver 1997; Bogosian et al. 1998). It has been suggested that elevated nutrients in resuscitation media may be toxic to VBNC cells (Whitesides and Oliver 1997). Non-growing cells can produce superoxide

and other free radicals on exposure to high nutrient levels, and such cells would not be able to detoxify these toxic radicals, which potentially results in preventing colony formation on media (Bloomfield et al. 1998). In the second group, the factors appear to have ability to repair damage or activate replicative functions (Yamamoto 2000). This has been described in studies on *Vibrio cholerae* (Wai et al. 1996) and *Helicobactor pylori* (Kurokawa et al. 1999) in which the non-culturable cells were recovered using media containing ammonium salts together with heat shock treatment. Similarly, the VBNC state of *E. coli* cells upon exposure to 0.8 M NaCl regained culturability after addition of 2 mM betaine (Na et al. 2006). Lastly, the third group of resuscitation factors is composed of those that act as signal molecules and are responsible for stimulation and regulation of physiological condition. Indeed, it has been reported that bacterial cells require the presence of adjoining cells or their products in certain conditions for their growth and survival (Yamamoto 2000; Arana et al. 2007). This has led to the proposal that the presence of culturable cells is a requirement, or at least plays an important role for the recovery of non-culturable cells. It is thought to be due to either the presence of the culturable cells themselves or their secretion of some signals or resuscitation-inducing factors that initiate the resuscitation process (McDougald et al. 1998; Ohtomo and Saito 2001). This idea cannot be confirmed by VBNC studies that involve pure cultures, since it would be not possible to determine whether the additional culturable cells were actually the product of growth of the culturable cells or resuscitated non-culturable cells (Bogosian et al. 1998; Bogosian and Bourneuf 2001). However, it is doubtful whether a culture without any culturable units is indispensable for VBNC studies (Ohtomo and Saito 2001), and the requirement for their presence in resuscitation may also help to explain the inconsistent results across those studies (McDougald et al. 1998).

While many bacteria have been reported to enter the VBNC state, very few studies have demonstrated true recovery of VBNC cells, as opposed to re-growth of culturable cells (Oliver 2005). One of the strong lines of evidence comes from the study of Whitesides and Oliver (1997) in which the VBNC population of *V. vulnificus* was extensively diluted to ensure the absence of any culturable cells. It has been demonstrated that resuscitation occurred at a faster rate when compared to the generation time of culturable cells. The study also observed that nutrient appears to inhibit the resuscitation of VBNC cells, suggesting that addition of nutrient in an attempt to initiate resuscitation is only able to detect potential presence of residual culturable cells (Whitesides and Oliver 1997). Demonstrating the resuscitation process has not always been a simple matter. Several studies argue that the recovered cell culturability is actually due to re-growth of an undetectable level of residual culturable cells hidden in the non-culturable population (Oliver 2005; Arana et al. 2007). This argument has arisen largely from the lack of stringent control in many early resuscitation studies (Kell et al. 1998). It is very important for VBNC studies to work with a culture without any culturable units. For this purpose, most studies tend to expose cells to a stressful environment for a long period (from 10 days to more than several months), or to achieve undetectable levels of culturable cells excluded by excessive dilution. However, there are still some difficulties with these approaches; long incubation periods make it difficult to obtain reliable and reproducible results, while excessive dilution to exclude culturable cells provides insufficient material for biochemical or molecular biological studies (Ohtomo and Saito 2001).

1.4.3 Significance of VBNC state in *E. coli*

Several lines of evidence have established that *E. coli* can form large populations of apparently non-culturable cells during exposure to stressful environments. Many of those reports have also indicated that *E. coli* often enter the VBNC state after prolonged incubation in aquatic environments such as seawater, ground water and rivers, especially at cold temperature (Xu et al. 1982; Rigsbee et al. 1997; Mizunoe et al. 1999; Ohtomo and Saito 2001; Na et al. 2006; Muela et al. 2008). Rigsbee et al. (1997) has demonstrated that three different strains of EHEC lose their culturability at 5°C in both river water and artificial sea water, but not at 25°C. A five strain cocktail of *E. coli* O157:H7 became non-culturable in both reservoir and lake water held at 25°C (Wang and Doyle 1998). Furthermore, it has been reported that the VBNC state of *E. coli* cells can be induced by conditions of high salt concentration (Ohtomo and Saito 2001). This supports the suggestions that *E. coli* O157 could enter the VBNC state in salted foods such as salted salmon roe (Makino et al. 2000).

Despite the above, there is no definite evidence showing that *E. coli* cells enter the VBNC state in foods and water (Bogosian and Bourneuf 2001), several studies have proposed potential formation of the VBNC cells linked with outbreaks (William et al. 1994; Mizunoe et al. 1999; Makino et al. 2000). One of those outbreaks involved the consumption of tap water contaminated with *E. coli* O157:H7 for more than a month. This prolonged outbreak was suggested to be due to the ability of *E. coli* to survive in lake water or repeated contamination, but it was not possible to recover the bacteria from the water samples. This failure was thought to have occurred because the cells may have entered the non-culturable state (Mizunoe et al. 1999). Therefore, the

VBNC state is considered to be a potential threat to public health, and it is very important to understand the physiological capacities of bacteria once they have entered such a state.

1.5 OBJECTIVE OF THIS THESIS

The ultimate aim of the work reported in this thesis is to provide a comprehensive understanding of the growth kinetics and cellular responses of *E. coli* O157:H7 strain Sakai subjected to conditions relevant to low temperature and water activity conditions experienced during meat carcass chilling in cold air. Such knowledge will aid the development of more targeted, and less invasive approaches, for the meat industry to eliminate or control this pathogen. The following objectives are presented to achieve the proposed aim:

1. to examine the growth kinetics of *E. coli* in response to a sudden downshift in temperature and/or water activity.
2. to assess the genomic and proteomic profiles of exponential phase *E. coli* under steady-state conditions of cold temperature and water activity stress.
3. to characterize the time-dependent alterations in gene expression and protein abundance of exponential phase *E. coli* upon exposure to a sudden downshift in temperature and/or water activity.

1.6 THESIS STRUCTURE

The structure of this thesis reflects the logical approach followed for the objectives of this research. Consequently, Chapters 2-5 are presented in a sequence, which allows them to build upon the findings of previous ones and, in some cases, investigate previously posed questions. All chapters have been prepared with the aims for submission to a peer-reviewed journal.

Because of this formatting and the multiple uses of similar approaches, there is some repetition within and between the chapters. However, each chapter has a unique focus and seeks to address a different set of aims, which relate to the physiological response of *E. coli* O157:H7 Sakai subjected to conditions relevant to low temperature and water activity conditions experienced during meat carcass chilling in cold air. More specifically, Chapter 2 provides a baseline of knowledge of the physiology of *E. coli* during balanced growth under cold temperature and water activity stress. This enables better interpretation, and potentially exploitation of its responses to dynamic environmental conditions that occur during carcass chilling. Chapters 3 and 4 offer an insight into the physiological responses of *E. coli* upon exposure to dynamic low temperature and water activity, respectively. To provide a complete picture of the effect of carcass chilling on *E. coli* physiology, Chapter 5 examines the potential mechanisms controlling population responses of *E. coli* after simultaneous abrupt downshifts in temperature and water activity and subsequent restoration of balanced growth.

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CHAPTER 2: INTEGRATED TRANSCRIPTOMIC AND PROTEOMIC ANALYSIS OF THE PHYSIOLOGICAL RESPONSE OF *ESCHERICHIA COLI* O157:H7 SAKAI TO STEADY-STATE CONDITIONS OF COLD AND WATER ACTIVITY STRESS

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CHAPTER 3

GLOBAL GENOMIC AND PROTEOMIC RESPONSES OF *ESCHERICHIA COLI* O157:H7 SAKAI DURING DYNAMIC CHANGES IN GROWTH KINETICS INDUCED BY AN ABRUPT TEMPERATURE DOWNSHIFT

3.1 INTRODUCTION

Refrigeration is the most common means of preserving food, either alone or in combination with other methods such as the addition of preservatives (Russell 2002). The ability to survive and grow at low temperature is, therefore, inherently important to many food-borne mesophilic bacterial pathogens, including *E. coli*. It has been well established that rapid transfers of an exponential phase *E. coli* from 37°C to a temperature below 20°C results in a transient arrest of cell growth for acclimation or adaption phase of one to several hours, depending on the magnitude of the shift, before resuming growth at a new rate which is slower than before the cold shock (Golovlev 2003; Gualerzi et al. 2003). During this cold acclimation phase, a number of physico-chemical changes occur, such as reduction in membrane fluidity, stabilization of the secondary structures of DNA and RNA, inefficient folding of some proteins, and translational block (Golovlev 2003; Phadtare 2004). Consequently, the bacterial cells induce an adaptive response to the cold shock stress, which involves major changes in gene expression and protein activity for the purpose of their survival. This cold shock response generally includes alterations in the physico-chemical state of cell membranes and changes in the transcriptional and translational machinery (Yamanaka 1999; Cao-Hoang et al. 2008). Furthermore, previous studies have reported that bacteria intentionally trended to have become adapted to the stress condition may develop resistance to other stress challenges (i.e. cross protection) (Aertsen and Michiels

2004; Chung et al. 2006). Therefore, this ability of pathogenic bacteria to adapt to and survive under stress conditions could increase the risk of food-borne illness. A comprehensive understanding of the molecular response of *E. coli* O157:H7 during adaptation and growth at low temperature is important in designing targeted processing and preservation strategies to effectively control this pathogen.

In chapter 2, an integrated transcriptomic and proteomic approach was used to determine the physiological response of *E. coli* O157:H7 strain Sakai during exponential growth phase under steady-state conditions relevant to low temperature and water activity conditions experienced during meat carcass chilling in cold air (Chapter 2; Kocharunchitt et al. 2011). The findings of that study provide a baseline of knowledge of the physiology of this pathogen, with the response of *E. coli* O157:H7 to steady-state condition of cold stress (14°C a_w 0.985), including activation of the master stress response regulator RpoS and the Rcs phosphorelay system involved in the biosynthesis of the exopolysacharide colanic acid, as well as down-regulation of genes and proteins involved in chemotaxis and motility (Kocharunchitt et al. 2011). To provide an insight into the genetic systems operating during exponential growth of *E. coli* O157:H7 Sakai in refrigerated foods, the study described in this chapter examined the growth kinetics of this pathogen during exposure to a sudden downshift in temperature from 35°C to either 20°C, 17°C, 14°C or 10°C, and extended the investigation into the time-dependent alterations in its genome and proteome upon cold shock from 35°C to 14°C. The global responses of *E. coli* were analyzed by both cDNA microarray and 2D-LC/MS/MS analyses. Differences in gene expression and protein abundance patterns in *E. coli* before and after cold shock were analyzed

through quantitative and comparative analysis of time series changes in both mRNA and protein levels.

3.2 MATERIALS AND METHODS

3.2.1 Division of experimental work

Growth experiments studying the response of *E. coli* to temperature downshift were conducted in the Food Safety Centre, University of Tasmania (Hobart, Australia). Parallel transcriptomic and proteomic studies were, however, undertaken in two independent laboratories, CSIRO Food and Nutritional Sciences (North Ryde, Australia) and the Food Safety Centre (Hobart, Australia), respectively. The differences in the protocol followed at each site are described in Section 2.2.1.

3.2.2 Bacterial strain

E. coli O157:H7 strain Sakai was used throughout this study (Section 2.2.2).

3.2.3 Abrupt downshift in temperature

A stationary phase culture of *E. coli* (Section 2.2.3) was diluted 1:10^4 in 25 ml of pre-warmed (35°C) brain-heart infusion (BHI) broth (CM225, Oxoid, Australia; Appendix A.7) to prepare a 'primary' culture. This culture was incubated at 35°C with agitation (80 oscillations.min^{-1}) in a water bath (Ratek Instruments, Australia), and its growth monitored turbidimetrically with a Spectronic 20 spectrophotometer (Bausch and Lomb, USA). After achieving an optical density at 600 nm (OD_{600}) of 0.1 ± 0.01 (i.e. the mid-exponential phase of growth, ~10^7 CFU.ml^{-1}), the culture was diluted 1:10^2 in a fresh batch of 25 ml of pre-warmed (35°C) BHI broth to prepare a 'secondary' culture. This culture was incubated at 35°C with agitation to the mid-exponential

growth phase (OD_{600} of 0.1 ± 0.01). At this point, the culture was abruptly shifted to a refrigerated water bath held either at 20°C, 17°C, 14°C or 10°C. Water baths were maintained within ± 0.1ºC of the test temperature, and were continuously logged using type T thermocouples connected to a Grant Squirrel, model 1255, data logger (Grant Instruments, Cambridge, UK).

3.2.4 Microbiological analysis

Growth of *E. coli* was determined by measuring percent transmittance (%T) at 600 nm using a Spectronic 20 spectrophotometer. The %T values were converted to OD values. All experiments were conducted at least twice, and the results are reported as the average of the replicates.

Growth curves were constructed by plotting the $\log_{10}$ OD *versus* time. Linear regression analysis of the growth curve data was then used to estimate generation time (GT) and lag time (LT) , according to the study of Mellefont et al. (2003). Briefly, a straight line was applied to fit data points that best represent exponential phase of growth. To ensure the accuracy of this estimation, at least four time points over this growth phase were used, and the minimum acceptable R^2 value for the regression line was 0.95. GT was then calculated by dividing 0.301 (equivalent to $\log_{10}$ 2) by the slope of the line. LT was estimated from the time at which the regression line through the exponential part of the growth curve first exceeded the initial cell numbers after applying the temperature downshift (i.e. the time taken to increase above starting numbers). To assess the relative amount of *work* required by *E. coli* to adjust to a new temperature, relative lag time (RLT) was calculated by dividing LT by GT (Robinson et al. 1998; Mellefont and Ross 2003).

3.2.5 Transcriptomic and proteomic analysis

In a separate set of cold shock experiments, *E. coli* cultures (five lots in total) were subjected to a rapid temperature downshift from 35°C to 14°C as described above (Section 3.2.3) to prepare samples for cDNA microarray and 2D-LC/MS/MS analysis. Each lot of the cell cultures (25 ml) was, respectively, harvested before temperature downshift (i.e. the reference culture), and at 30, 90, 160, and 330 min after the shift. Independent samples were then extracted for RNA, and both soluble and membrane proteins as detailed in Sections 2.2.7 and 2.2.10, respectively. It should be noted that the method used to extract soluble and membrane proteins was slightly modified in that both fractions were sequentially extracted from the same sample (rather than separate samples as described in Section 2.2.10). The number of biological replicates taken at each time point for the transcriptomic and proteomic analysis is given in Table 3.1.

Table 3.1 Number of biological replicates performed in cDNA microarray and 2D-LC/MS/MS analysis.

Time points	Number of biological replicates	
	Transcriptome	Proteome[a]
Before temperature downshift	3	6(6)
30 min after shift	3	2(2)
90 min after shift	3	2(2)
160 min after shift	3	2(2)
330 min after shift	3	2(2)

[a] Number of replicates performed for soluble (outside brackets) and membrane (within brackets) fractions of *E. coli*.

All mRNA and protein samples were analyzed using analysis of cDNA microarray (Section 2.2.8) and 2D-LC/MS/MS (Section 2.2.12), respectively.

3.2.6 Microarray data analysis

Microarray data were subjected to background correction, normalization and calculation of the expression measures according to Section 2.2.9. Significance analysis was conducted using one way analysis of variance (ANOVA) using the multiple correction testing method of Benjamini and Hochberg (1995) with a *P*-value cut-off of <0.01. Elements were considered to be differentially expressed if the fold change was >2.

3.2.7 MS/MS data analysis

MS/MS spectra obtained were processed and searched against the complete database of *E. coli* O157:H7 Sakai (5,318 entries in total) as described in Section 2.2.13. The confidence level of protein identifications was then assigned with the following modifications. All protein identifications that passed the criteria (i.e. a PeptideProphet and ProteinProphet of ≥0.9) were further assessed based on their confidence level across biological replicates of both membrane and soluble fractions of *E. coli* cells at each time point. The confidence level was based on the number of unique peptides identified from one sample and the number of replicates in which the protein was detected. Specifically, proteins identified by more than one unique peptide in at least one of the replicates were considered to have a 'high' confidence score. The 'intermediate' confidence level was assigned to proteins with a single peptide hit that was detected in more than one replicate. 'Low-confidence' proteins were considered to be those identified by a single unique peptide and found in only one replicate. Only protein identifications with a 'high' and 'intermediate' confidence level (referred as having a 'high confidence score') were accepted for further analysis.

Following data filtering, all protein identifications with high confidence from membrane and soluble fractions of the same sample were combined to represent a 'total' fraction of the corresponding sample. This combined approach was employed on the basis that the observed R^2 values, as determined by linear regression analysis, indicated a relatively stronger linear correlation between spectral counts (SpCs, the number of MS/MS spectra) for all possible pair-wise comparisons of replicates of pooled fractions (average $R^2 = 0.89 \pm 0.05$) when compared to that of replicates of each fraction (average $R^2 = 0.64 \pm 0.19$, and 0.85 ± 0.05 for membrane and soluble fractions, respectively). Each of the total fractions across the biological replicates was then used to generate the final list of proteins identified at each sampling time.

3.2.8 Protein abundance ratio and its significance

Spectral count generated by 2D-LC/MS/MS analysis was used as a semi-quantitative measure of protein abundance (Nesvizhskii et al. 2003; Liu et al. 2004). The total abundance of each protein in a sample was obtained by pooling spectral counts detected for a given protein from membrane and soluble fractions of the same sample. Each pooled spectral count was then normalized to obtain the normalized spectral abundance factor (NSAF) according to Zybailov et al. (2006). Briefly, the NSAF for a given protein is the pooled spectral count of the protein divided by its length (L), and normalized against the average SpC/L across all proteins in the corresponding dataset. The NSAF values were then averaged across all biological replicates at each time point of sampling.

Fold changes in protein abundance due to cold shock were calculated as the $\log_2$ ratio of the average NSAF value in the cold-treated sample over the average NSAF value of the control. To

avoid errors during fold-change calculation in cases where a zero value was obtained, a correction factor of 0.01was added to each of the average NSAF values. Statistical analysis of differences in protein abundance was then carried out using the beta-binomial test implemented in R (Pham et al. 2010). All proteins with a *P*-value <0.01 and at least 2-fold change were considered to be differentially abundant.

3.2.9 Transcriptomic and proteomic data mining

Information on identified genes and proteins such as protein and gene names, ECs numbers (locus tag), GI numbers, NCBI Reference Sequence (RefSeq), protein sizes and molecular masses were obtained from the UniProt knowledgebase (http://www.uniprot.org/, accessed on 5.12.2010) and the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/, accessed on 5.12.2010). Preliminary functions and properties of the genes and proteins were based on the EcoCyc database (http://www.ecocyc.org/, accessed on 13.12.2010), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/, accessed on accessed on 4.12.2010). In the present study, protein or gene names in conjunction with ECs numbers were used as a unique identifier for identified genes and/or proteins.

3.2.10 Predefined set enrichment analysis

To determine changes in overall expression of a predefined set of genes or proteins due to temperature downshift, the T-profiler analysis was performed on the $\log_2$ ratios according to Section 2.2.16. The predefined sets of genes or proteins used for T-profiler analysis were based on the selected functional role categories and/or metabolic pathways of the following databases:

the JCVI Comprehensive Microbial Resource (JCVI CMR) database (http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi, accessed on 5.12.2010), the EcoCyc database collection (http://www.ecocyc.org/, accessed on 13.12.2010), and the KEGG database (http://www.genome.jp/kegg/, accessed on 4.12.2010), as well as based on the lists of genes or proteins previously known to be positively controlled by major regulons, including CpxRA (Price and Raivio 2009), RpoE (Rhodius et al. 2006), RpoH (Nonaka et al. 2006), RpoS (Lacour and Landini 2004; Patten et al. 2004; Weber et al. 2005), and the Rcs phosphorelay system (Hagiwara et al. 2003). The *T*-value obtained from the analysis was determined only for sets that contained at least five members, and its significance was established by using the associated two-tailed *P*-value. All predefined sets with a *P*-value less than 0.1 were considered to be statistically significant.

3.3 RESULTS AND DISCUSSION

3.3.1 Growth of *E. coli* O157:H7 Sakai upon temperature downshift

The growth response of *E. coli* upon temperature downshift of exponential phase cultures in a nutrient rich medium was examined. Specifically, cultures were exposed to a sudden downshift in temperature from 35°C to either 20°C, 17°C, 14°C or 10°C. It was found, from the turbidimetric data, that all downshifts caused a lag period before growth resumed at a slower rate (Fig. 3.1). Similarly, this pattern of growth response was also observed using viable count data obtained in parallel, but at less sampling times (see Appendix D for more details).

Estimates of growth kinetics for each temperature downshift are summarized in Table 3.2. The generation times obtained were consistent with the predictive model of Ross et al. (2003), and

both generation and lag times increased with the magnitude of the shift. However, the RLT response for *E. coli* showed no systematic variation across the temperature range tested ($y = -0.02x + 0.03$, $R^2 = 0.33$), indicating that the amount of *work* used to adapt to each temperature was similar (Table 3.2). This agrees well with the study of Mellefont and Ross (2003), demonstrating that shifting *Klebsiella oxytoca* cells from a temperature within the normal physiological range to temperatures outside this range did not appear to have an effect on RLT. The normal physiological range for temperature of *E. coli* is estimated to be from approximately 20°C to 38°C (Golovlev 2003; Mellefont and Ross 2003; Giuliodori et al. 2007). Further investigations involving larger downshifts together with validation of the data by culture-independent methods, are still required to better characterize the RLT response of *E. coli* O157:H7 Sakai to temperature downshift and to confirm the present findings.

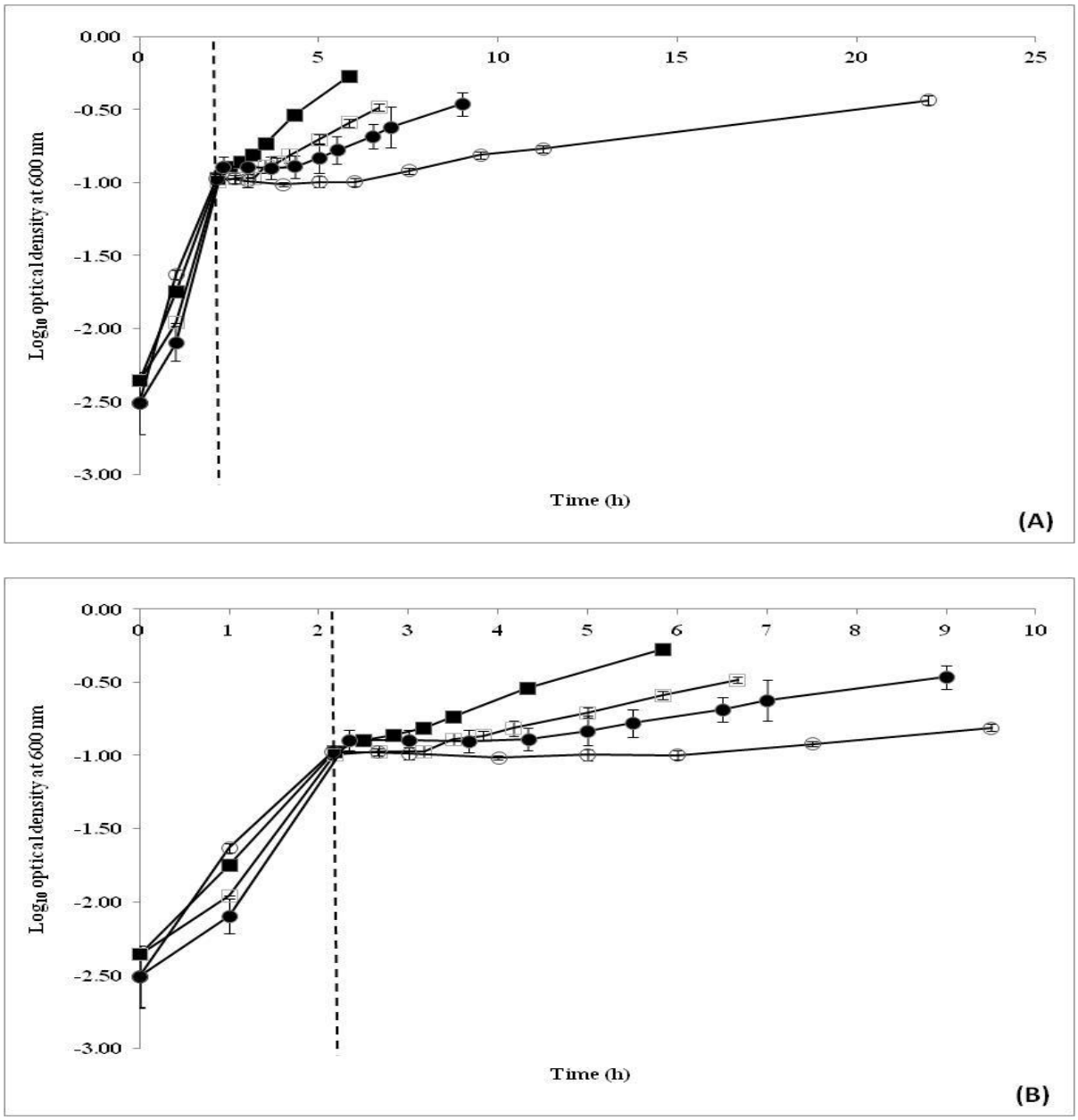

Fig. 3.1. Growth response of exponential phase *E. coli* O157:H7 Sakai to a rapid downshift in temperature from 35°C to 20°C (■), 17°C (□), 14°C (●) and 10°C (○), as determined by optical density. The time at which cold shock was applied is indicated by a dotted line. Data points represent means ± standard deviations of at least two independent replicates. Panel (A) shows all available data and panel (B) shows all data on a reduced time scale.

Table 3.2 Summary of the growth response of exponential phase *E. coli* O157:H7 Sakai upon an abrupt temperature downshift from 35°C to either 20°C, 17°C, 14°C or 10°C, as determined by optical density.

Temperature downshift	Generation time, GT (h)[a]	Lag time, LT (h)[b]	Relative lag time (RLT)
20°C	1.29	0.30	0.23
17°C	1.93	0.84	0.44
14°C	3.28	1.95	0.59
10°C	9.01	3.81	0.42

[a] Generation time (GT) is determined by linear regression

[b] Lag time (LT) is calculated by linear regression as the time taken to increase above starting numbers.

3.3.2 Molecular response of *E. coli* O157:H7 Sakai to cold shock

To reveal the entire genetic system that operates during exposure to chill temperature, the present study further employed both transcriptomic and proteomic approaches in parallel to characterize temporal alterations in mRNA expression and protein production in *E. coli* upon a sudden downshift in temperature from 35°C to 14°C (Fig. 3.1). Samples taken for transcriptomic and proteomic analysis were taken during cold adaptation (30 and 90 min after a temperature downshift) and in cold-adapted cells (160 and 330 min post-temperature downshift) (Fig. 3.2). Independent samples were then processed for RNA, and membrane and soluble protein extractions as appropriate for analysis of cDNA microarray and 2D-LC/MS/MS, respectively. Through multidimensional LC/MS/MS analysis together with data filtering, several protein identifications with high confidence were detected in total fractions of *E. coli* at each time point (Appendix E.1 and Table 3.3). It was also found that an average of 19.1% ± 7.1% of these proteins across all proteomic datasets was uniquely identified from the membrane fraction. This indicates that additional extraction of membrane fractions moderately improved proteome coverage. In addition, the peptide false-positive discovery rate as determined by dividing the number of spectra matching decoy peptides with the total number of spectra was estimated for each dataset, and shown to be less than 5% for all MudPIT runs (Appendix E.2).

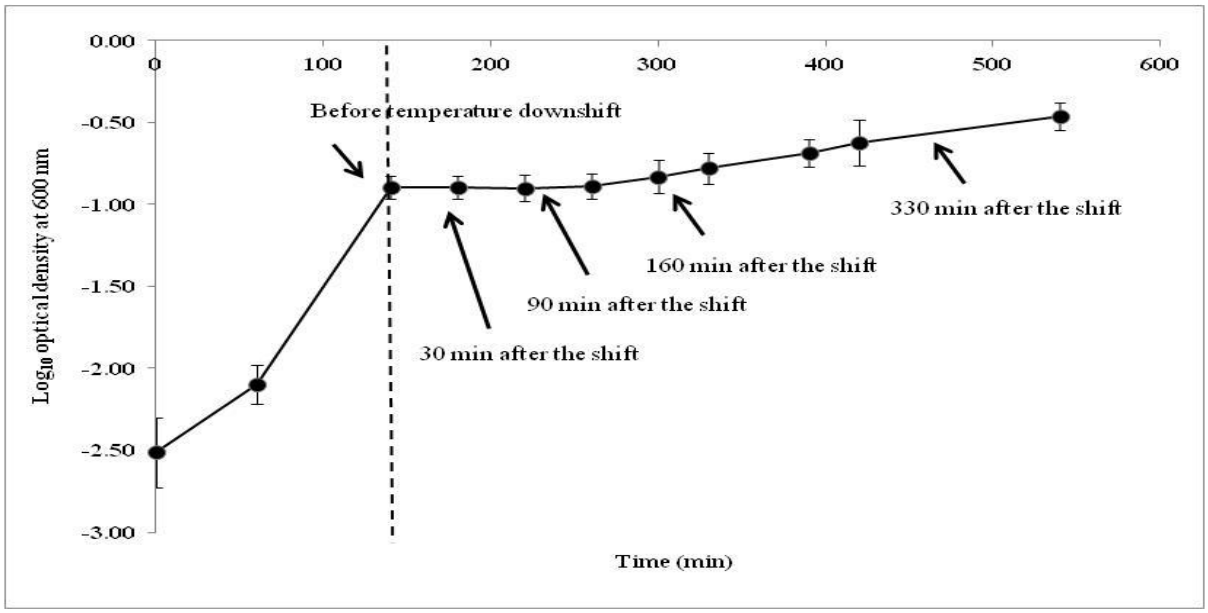

Fig. 3.2. Sampling scheme for analysis of molecular response of *E. coli* O157:H7 Sakai to a temperature shift from 35°C to 14°C. The time at which temperature downshift was applied is indicated by a dotted line. Block solid arrows indicate the sampling point for cDNA microarray and 2D-LC/MS/MS analyses. Data points represent means ± standard deviations of at least two independent replicates.

Table 3.3 The number of protein identifications and differentially expressed genes and proteins during exposure of *E. coli* O157:H7 Sakai to cold stress.

Time points	Number of proteins			Number of differentially expressed elements[d]		
	Membrane fraction[a]	Soluble fraction[a]	Total fraction[b]	Trancriptome (total)[e]	Proteome (total)	Transcriptome *vs.* Proteome
Before temperature downshift	343	1,281	1,225(61)[c]	NA[f]	NA	NA
30 min after shift	704	824	921(213)	503(292)	137(20)	13(3)
90 min after shift	717	864	968(214)	637(466)	155(26)	26(2)
160 min after shift	702	890	970(184)	594(378)	118(25)	22(6)
330 min after shift	858	864	1,025(247)	496(301)	201(63)	30(7)

[a] Number of proteins identified in membrane and soluble fractions of the *E. coli* proteome that pass the filtering criteria (i.e. at PeptideProphet and ProteinProphet of ≥0.9).

[b] Number of protein identifications with high confidence in total fraction of the *E. coli* proteome (outside brackets).

[c] Number of high-confidence proteins uniquely identified in membrane fraction (within brackets).

[d] Number of elements with increased (outside brackets) and decreased (within brackets) expression.

[e] Analysis does not include the number of differentially expressed undefined intergenic regions in the transcriptome.

[f] NA; not applicable.

Gene expression and protein abundance profiles at each of these time points were compared with those of a control culture at 35°C (i.e. reference sample). The number of differentially expressed genes and proteins at each time point are shown in Table 3.3. It was found that the number of up-regulated elements was dominant over those with decrease in expression level at all time points. This is consistent with a previous time course study examining the genome-wide transcriptional alterations in *Vibrio parahaemolyticus* upon sudden temperature downshift (Yang et al. 2009). A comprehensive list of differentially expressed genes and proteins with annotations, as well as their comparison to previously published data is given in Appendix E.3. Furthermore, the transcriptomic and proteomic profiles were compared for each time point. The level of correlation, as determined by calculating the percentage of differentially expressed genes and proteins matched was found to be low, with between 0.4% and 6.1% of elements matched across all time points (see Appendix E.4). This was despite that a number of consistent trends were still observed in the data, as discussed below.

E. coli exposed to a rapid downshift in temperature showed expression changes of different groups of genes and proteins, as determined by the T-profiler analysis (For full results see Appendix E.5). These genes and proteins are known to be associated with a number of functional categories and metabolic pathways, as well as play an important function in the stress responses that are controlled by regulons, as discussed below.

3.3.2.1 DNA metabolism

The requirement to conserve energy is an important feature of all stress responses (Weber et al. 2005; Durfee et al. 2008; Jozefczuk et al. 2010). The implementation of the RpoS-dependent

general stress response (activation of the RpoS regulon also observed here; see Section 3.3.2.8) has previously been shown to reduce energy expenditure through the repression of genes involved in growth, cell division, and protein synthesis (Weber et al. 2005). In the present study, the T-profiler analysis revealed a significant decrease in overall abundance of proteins involved in DNA replication, recombination, and repair at all time points, except at 90 min after imposition of the cold stress (Fig. 3.3). A number of these proteins were found to be significantly down-regulated after 30 (MutS and TopA), 90 (MutS), 160 (MutS, StpA and TopA) and 330 (MutS and RecA) min of imposition of the cold stress. Interestingly, gene encoding DNA mismatch repair protein (*mutS*) also exhibited a significant down-regulation at all time points. It has been reported that production of MutS decreases during stationary phase. This pattern of regulation is thought to reflect a mechanism in which cells coordinate the amount of MutS with decreases in the level of DNA replication (Tsui et al. 1997). The apparent down-regulation of *mutS*/MutS may, therefore, reflect changes in the expression of genes and proteins involved in transcription and translation. In support of this, Li et al. (2003) has suggested that decrease in production of MutS may contribute significantly to increased mutation or homologous recombination within populations in environmental stresses. This possibly allows the evolution of *E. coli* cells better to be able to grow at low temperature in the present study.

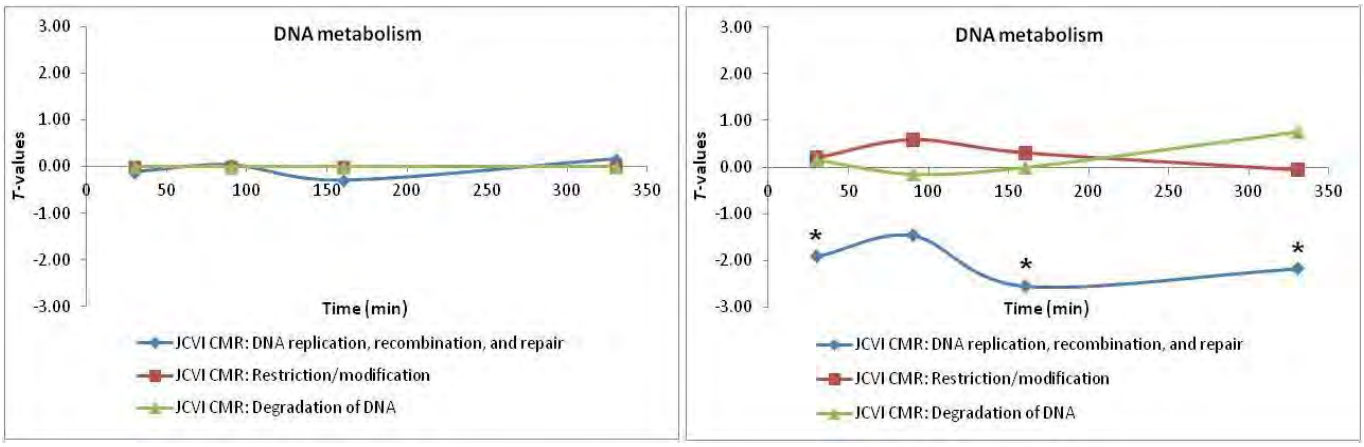

Fig. 3.3. Changes in overall expression of genes (left panel) and proteins (right panel) involved in DNA metabolism during exposure of *E. coli* O157:H7 Sakai to temperature downshift from 35°C to 14°C, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated database, as described in Section 3.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

3.3.2.2 Transcription and translation

As the cultivation temperature decreases after temperature shift, the corresponding growth rate decreases, and so should the rate of protein synthesis, DNA replication and the need for nucleotides. One of the major physiological effects of cold temperature is also the stabilization of secondary structures of RNA and DNA, which may affect the efficiencies of translation, transcription, and replication (Polissi et al. 2003). In keeping with this, the present study observed significant negative *T*-values for elements involved in several functional categories of purines, pyrimidines, nucleosides and nucleotides, and those of protein synthesis (Fig. 3.4). These included those genes involved in 2' deoxyribonucleotide metabolism (at all time points); genes involved in the salvage of nucleosides and nucleotides (at all time points); genes (within the first 160 min) and proteins (at time 90 min) involved in purine ribonucleotide biosynthesis; genes involved in tRNA aminoacylation at all time points; genes (at 90 and 160 min of the cold

stress) and proteins (from 90 min onward) involved in tRNA and rRNA base modification; and genes involved in the synthesis and modification of ribosomal proteins (at time 30 and 330 min).

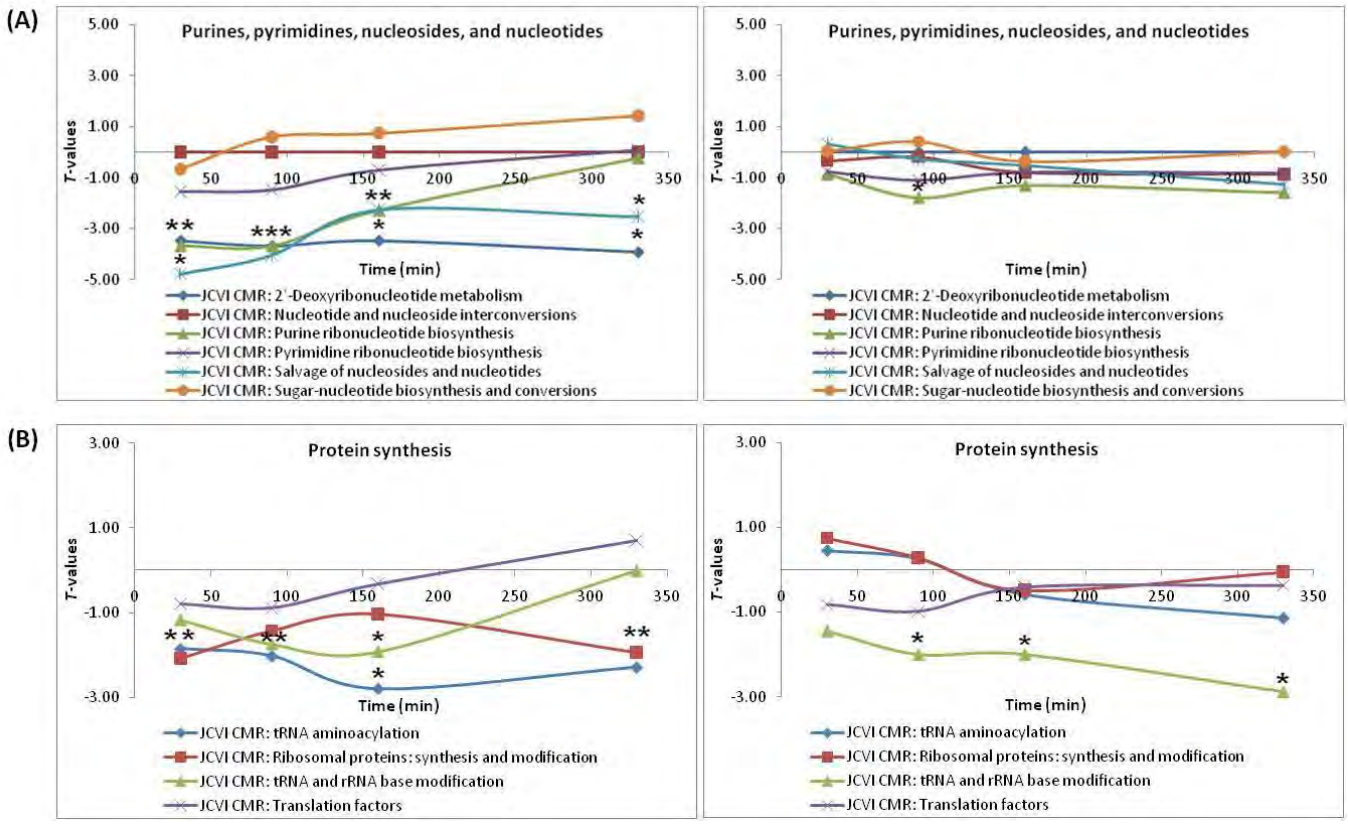

Fig. 3.4. Changes in overall expression of genes (left panel) and proteins (right panel) involved in functional categories of purines, pyrimidines, nucleosides and nucleotides (A) and protein synthesis (B) during exposure of *E. coli* O157:H7 Sakai to temperature downshift from 35°C to 14°C, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated database, as described in Section 3.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

3.3.2.3 Carbohydrate catabolism and energy generation

Genes involved in glycolysis (at 30 and 90 min after temperature downshift), gluconeogenesis (at time 30 min), the pentose phosphate pathway (at time 30 min), mixed acid fermentation (at time

30 min), and oxidative phosphorylation (within the first 160 min) exhibited a decrease in expression with significant negative *T*-values (Fig. 3.5). This initial down-regulation of genes involved in carbohydrate catabolism corresponds well with the observed down-regulation of sugar transporters (see Section 3.3.2.5). By contrast, the proteomic analysis revealed a significant increase in overall abundance of proteins involved in oxidative phosphorylation throughout the course of cold stress. This may indicate a perturbation in respiratory balance during exposure to such stress.

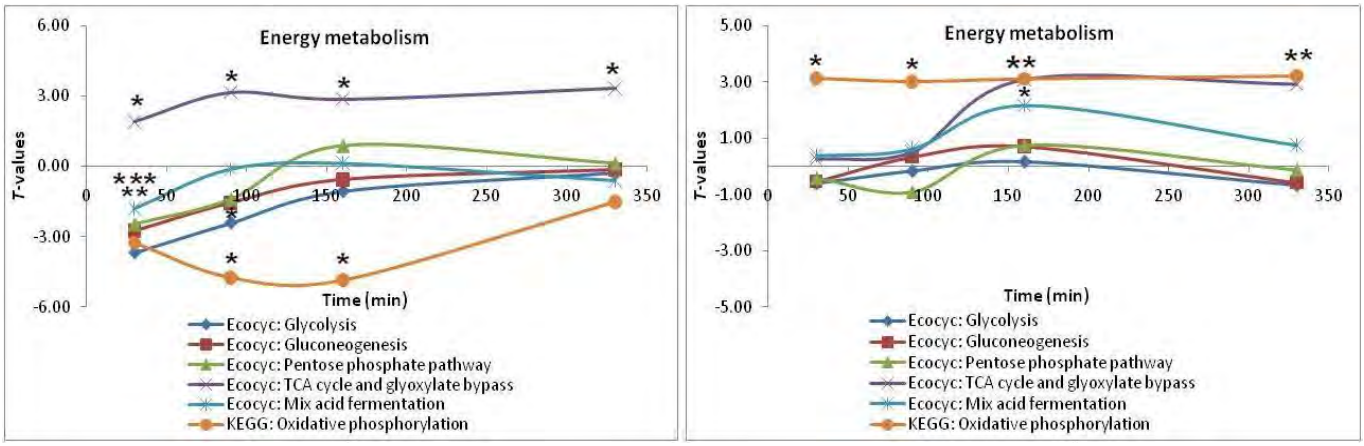

Fig. 3.5. Changes in overall expression of genes (left panel) and proteins (right panel) involved in energy metabolism during exposure of *E. coli* O157:H7 Sakai to temperature downshift from 35°C to 14°C, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated databases, as described in Section 3.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

TCA cycle and glycoxylate bypass genes were up-regulated at all time points. However, this trend was apparent in the proteomic data only from 160 min after the shift (Fig. 3.5). This observation corresponds well with previous findings in which temperature downshift resulted in increased expression levels of genes encoding enzymes in the TCA cycle to compensate for the

reduction in activity of TCA cycle enzymes at lower temperatures (Gadgil et al. 2005). However, oxygen consumption rates have also been shown to be lower at lower temperatures and it has been suggested that this points towards a reduced carbon flux through the TCA cycle and a reduced electron flux through the electron transport pathway (Gadgil et al. 2005). In the present study, the T-profiler analysis of the transcriptomic data also revealed down-regulation of electron transport-associated genes at 30 (*ykgC*, *yddW*, *dusC*, *ccmE*, *nuoBCF*, *hmp* and *uxuB*), 90 (*kefF*, *ykgC*, *cydAB*, *trxB*, *yddW*, *sufBD*, *yedF*, *yeeD*, *dusC*, *ccmCEFH*, *napGB*, *yfaE*, *nuoABCEFHIJKLM* and *hmp*) and 160 (*cydABC*, *nuoHIJKLMN*, *ccmCEFH, trxB*, *ndh*, *yddW*, *yeeD*, *dusC*, *napC*, *yfaE*, *hmp*, *yigI* and *ycaO*) min of the cold stress.

3.3.2.4 Amino acid biosynthesis

A number of amino acid biosynthetic pathways were negatively regulated after a shift to chill temperature (Fig. 3.6). In addition, genes encoding tRNA synthetases were down-regulated at 30 (*aspS*, *argS*, *gltX*, *lysS* and *glyS*), 90 (*tyrS*, *pheS*, *aspS*, *argS*, *gltX*, *alaS*, *lysS*, *trpS* and *yadB*), 160 (*pheST*, *argS*, *gltX*, *lysSU*, and *yadB*) and 330 (*lysU* and *yadB*) min after temperature downshift. Analysis of the proteome data only revealed a significant down-regulation of prolyl-tRNA synthetase (ProS) at 330 min. In keeping with the study on the cold shock response of *Bacillus subtilis* (Beckering et al. 2002), the slower growth rate observed after a shift to 14°C resulted in a reduced capacity of protein synthesis, which may lead to an oversupply of amino acids and feedback inhibition of genes associated with amino acid biosynthesis. A similar observation was also made for the specific amino acid biosynthesis families down-regulated in the present study and in a previous report on the transcriptional response of *E. coli* to temperature downshift

(Gadgil et al. 2005) in which significant negative *T*-values were observed for amino acid biosynthesis in the aspartate and the pyruvate families (Fig. 3.6).

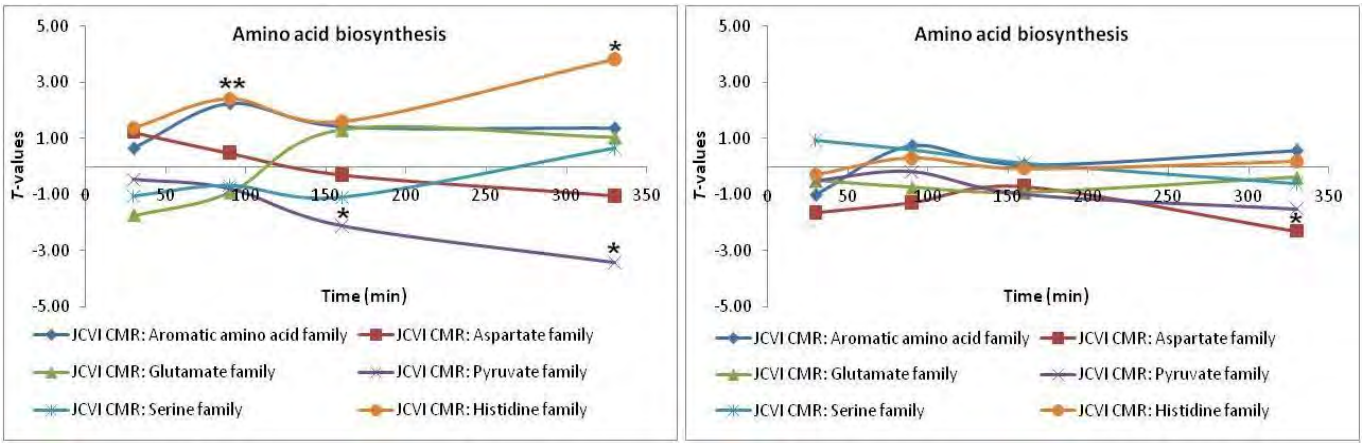

Fig. 3.6. Changes in overall expression of genes (left panel) and proteins (right panel) involved in amino acid biosynthesis during exposure of *E. coli* O157:H7 Sakai to temperature downshift from 35°C to 14°C, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated database, as described in Section 3.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

It is well established that the activation of specific amino acid biosynthesis pathways or amino acid supplementation can lead to tolerance to certain environmental stresses (Horinouchi et al. 2010). In a report examining the thermal adaptation of *Exiguobacterium sibiricum*, a psychrotrophic bacterium isolated from 3 million year old Siberian permafrost that grows from -5°C to 39°C, it has been demonstrated that cold stress leads to flux and pool size redistribution throughout the entire network of amino acid metabolism (Rodrigues et al. 2008). Rodrigues et al. (2008) suggested that the reason for a change in cell metabolism may be to synthesize more 'flexible' proteins that can function at lower temperatures. In this study, the positive regulation of specific amino acid biosynthesis pathways was in keeping with previous reports of the

response of *E. coli* to chill temperature with positive *T*-values for the histidine family at 90 and 330 min of the cold stress (Jozefczuk et al. 2010; Kocharunchitt et al. 2011) and positive *T*-values for amino acid biosynthesis in the aromatic amino acid family at time 90 min with the significant up-regulation of genes involved in the biosynthesis of phenylalanine (*pheA* and *aroG*), tyrosine (*pheA*), tryptophan (*trpEDCBA*) and chorismate (*aroLE*) (Kocharunchitt et al. 2011). Deciphering the role histidine and aromatic amino acids play under these test conditions would provide insight into whether the synthesis of these amino acids is vital for adaptation and growth at chill temperature.

3.3.2.5 Transport and binding proteins

Cold-induced remodelling of the bacterial cell envelope can affect the transport/binding functions that are essential for accumulation of nutrients against a concentration gradient, as well as for the excretion of various end products of metabolism (Yang et al. 2009). Cold shock has been shown to result in extensive alterations of the cell envelope structure and transmembrane transporting functions (Yang et al. 2009). In keeping with this, the present study observed numerous changes in expression patterns of genes and proteins involved in transport and binding (Fig. 3.7). It was of interest that an increase in the abundance of proteins involved in the transport of amino acids, peptides and amines was observed at all time points. A number of proteins responsible for the uptake of iron, osmoprotectants proline and glycine betaine, and uncharacterized substrates also exhibited a significant up-regulation at 30 (FhuC and YojI), 90 (FhuC, YojI, YadG and ProV), 160 (FhuC, YojI and ProVW) and 330 (FhuC, YojI, ProVW, YadG, ProP and YehZ) min of the cold stress. It is well established that the chill stress response in bacteria includes the uptake and synthesis of compatible solutes (Ko and Smith 1999;

Angelidis and Smith 2003; Ozcan et al. 2005), and proline transporters have previously been shown to be up-regulated upon exposure of *E. coli* to reduced temperature (Gadgil et al. 2005).

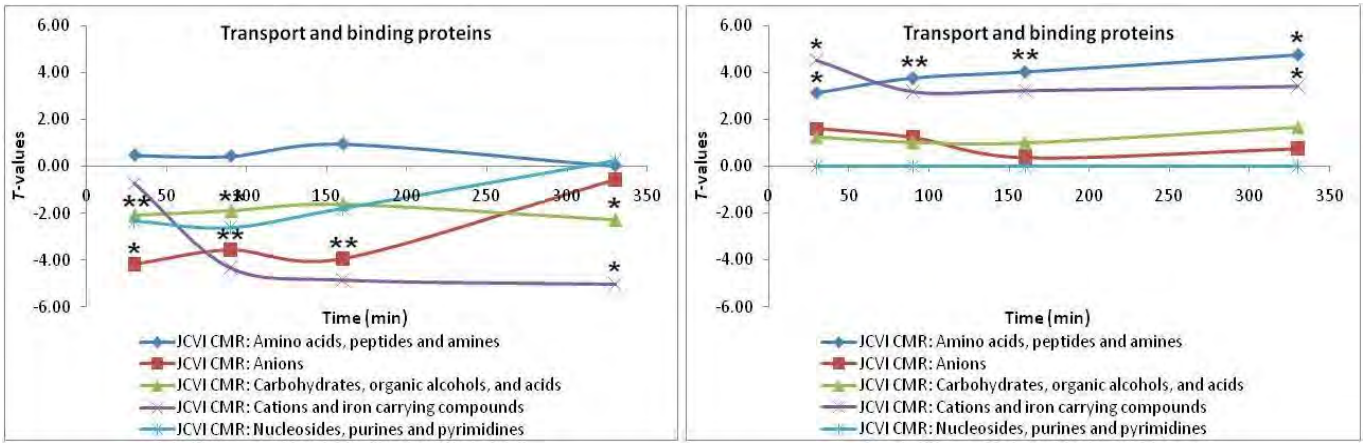

Fig. 3.7. Changes in overall expression of genes (left panel) and proteins (right panel) involved in transport systems during exposure of *E. coli* O157:H7 Sakai to temperature downshift from 35°C to 14°C, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated database, as described in Section 3.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

A contrasting expression pattern was observed for elements involved in the transport of cations and iron-carrying compounds, with the down-regulation of genes (from 90 min after cold shock onward) and up-regulation of proteins (at all time points) (Fig. 3.7). The proteomic data revealed a significant increase in abundance of a putative outer membrane receptor for iron transport (Fiu) and a magnesium ion transporter (CorA) at all time points, whereas it was evident in the transcriptomic data that expression of *fiu* (from 90 min of cold stress onward) and *corA* (at time 90 min) was significantly reduced.

The transcriptomic data also revealed a significant decrease in expression of several genes involved in anion transport within the first 160 min of the cold stress (Fig. 3.7). These included *cysPUWA*, *gntU* and *pstSCA*, encoding transporters for sulfate, gluconate and inorganic phosphate.

Expression of genes involved in the transport of carbohydrates, organic alcohols and acids were reduced with significant negative *T*-values at 30, 90 and 330 min after cold shock (Fig. 3.7). The majority of these down-regulated genes at time 30 (*manXYZ*, *malE*, *treB*, *uhpA*, *ptsG*, *ptsHI*, *gntU*, *yhjX*, *mtlA* and *mglA*), 90 (*manXYZ*, *malE*, *treB*, *uhpABC*, *ptsG*, *ptsHI*, *gntU*, *yhjX*, *mtlA*, *focA*, *etk*, *fadL* and *rbsA*) and 330 (*manX*, *malE*, *treB*, *uhpABC*, *uhpT*, *malF* and *dcuA*) min appeared to be associated with the transport of sugar (i.e. mannose, maltose, trehalose, glucose, galactose, ribose). The apparent down-regulation of these sugar transport genes coincides with the observation that genes involved in carbohydrate catabolism exhibited a decrease in expression (section 3.3.2.3).

Genes involved in the transport of nucleosides, purines and pyrimidines were significantly down-regulated at 30 and 90 min (Fig. 3.7). However, Tsx, which is the high affinity uptake system for ribo- and deoxy-nucleosides across the outer membrane, was highly up-regulated at all time points.

3.3.2.6 Fatty acids and lipids metabolism

Exposure of bacterial cells to cold shock or growth at low temperature can have effects on the outer membrane lipid composition that are beneficial to the cell (Vorachek-Warren et al. 2002).

It has been shown that palmitoleate is not present in lipid A isolated from *E. coli* cells grown above 30°C, but has been shown to comprise ~11% of the fatty acid acyl chains of lipid A in cells grown at 12°C (Carty et al. 1999). Consistent with this, the transcriptomic analysis revealed a significant increase in expression of *lpxP* encoding palmitoleoyl transferase at all time points except at time 160 min of the cold stress. The proteomic analysis, however, did not show this pattern.

It is well established that a decrease in growth temperature leads to an increased proportion of unsaturated fatty-acid residues in microbial lipids, resulting in a lowering of the melting point of the lipids (Russel et al. 1995; Yamanaka 1999). However, the expression of genes and proteins involved in fatty acid metabolism was not significantly affected upon temperature shift in the present study (Fig. 3.8). The ratio of saturated to unsaturated fatty acids is controlled by the relative levels of FabA and FabB, with overproduction of FabA leading to an increase in synthesis of saturated fatty acids (Clark et al. 1983). In keeping with the study of Gadgil et al. (2005), the expression level of *fabA*/FabA remained unchanged throughout the course of cold stress, although a significant up-regulation of *fabB*/FabB was not evident in this study. Expression of *sfa* was, however, significantly induced at all time points. It has previously been shown that Sfa increases the biosynthesis of unsaturated fatty acids, presumably by increasing the activity of FabB (Rock et al. 1996).

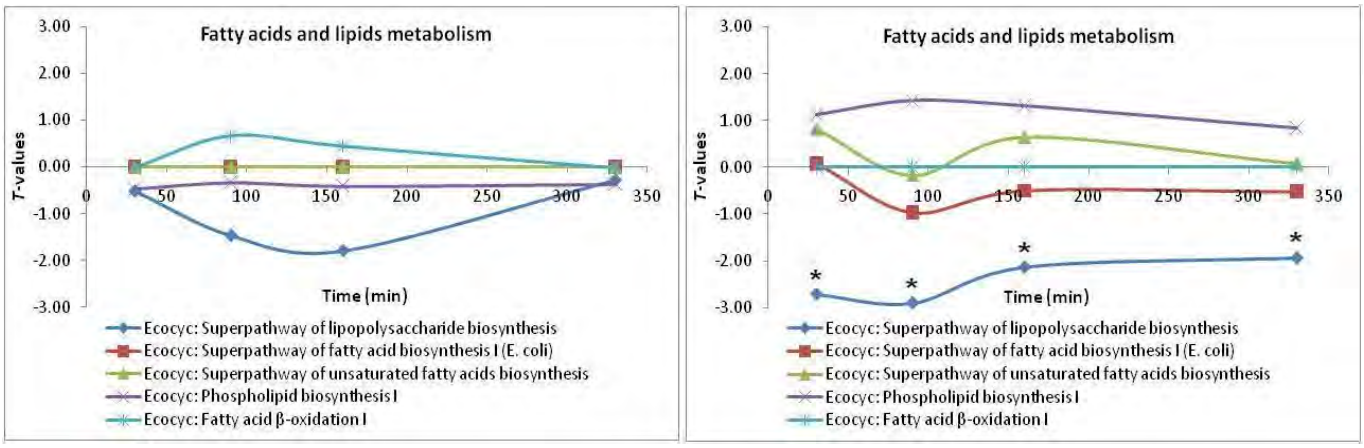

Fig. 3.8. Changes in overall expression of genes (left panel) and proteins (right panel) involved in fatty acids and lipids metabolism during exposure of *E. coli* O157:H7 Sakai to temperature downshift from 35°C to 14°C, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated database, as described in Section 3.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

Diffusion rates are typically reduced at low temperature and the outer membrane diffusion pore proteins OmpF and OmpC are thermoregulated in an inversely related manner. The larger of these two pores, OmpF, is expressed at an elevated level at cold, compared to optimal growth temperatures. At the same time, the amount of OmpC is reduced. In the present study, expression of *ompF*/OmpF was significantly induced at 330 min of the cold stress (Appendix E.3). This was despite that a significant increase in abundance of OmpC was observed at time 90 and 330 min.

3.3.2.7 Bacterial chemotaxis and motility

Genes involved in chemotaxis and motility were significantly down-regulated from 90 min after temperature downshift onward, as indicated by significant negative *T*-values (Fig. 3.9). This coincides with previous findings in which chemotaxis and motility genes and proteins were down-regulated during steady-state growth at 14°C a_w 0.985 (Kocharunchitt et al. 2011). The

apparent down-regulation of flagellar genes has also been reported in *E. coli* during shift to cultivation temperatures of 15°C (Moen et al. 2009) and 16°C (Jozefczuk et al. 2010), as well as in *B. subtilis* during growth at low temperature (Budde et al. 2006). Expression of genes encoding flagella are negatively controlled by the master regulator RpoS (Patten et al. 2004) and the Rcs system-activated colanic acid biosynthesis (Francez-Charlot et al. 2003).

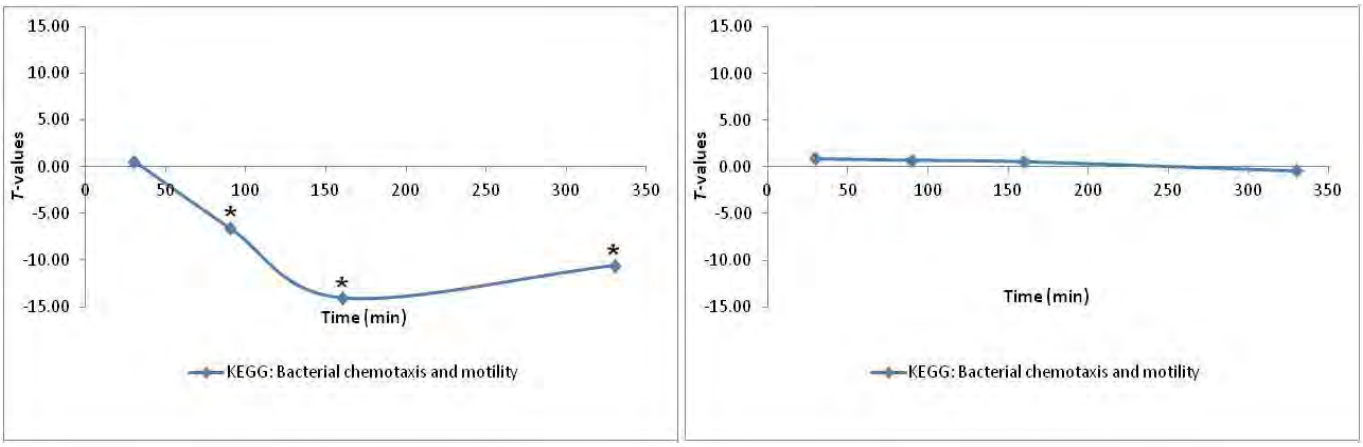

Fig. 3.9. Changes in overall expression of genes (left panel) and proteins (right panel) involved in bacterial chemotaxis and motility during exposure of *E. coli* O157:H7 Sakai to temperature downshift from 35°C to 14°C, as determined by T-profiler analysis. The predefined set used for T-profiler analysis was based on the indicated database, as described in Section 3.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

3.3.2.8 Stress response

Among members of the CspA family (from CspA-CspI), CspA, CspB, CspG and CspI are termed the major cold shock proteins in *E. coli* on the basis of their levels of induction upon cold shock (Yamanaka 1999). Increased production of these proteins has been reported to be essential for bacterial cells to resume growth at low temperature (Thieringer and Jones 1998; Yamanaka 1999). In the present study, two of these cold shock proteins exhibited a significant up-regulation

during exposure to cold stress, including *cspA* (at time 30, 90 and 330 min), CspA (from 90 min onward) and *cspG*/CspG (at all time points). Such expression pattern of these elements is inconsistent with previous proteomic studies, which reported that they are up-regulated during the acclimation phase upon cold shock and subsequently down-regulated when cells have become cold-adapted (Jones et al. 1987; Thieringer and Jones 1998; Etchegaray and Inouye 1999). This difference in observations might be due to the different approaches used in this study (i.e. using cDNA microarray and 2D-LC/MS/MS analyses to examine changes in gene expression and protein abundance profiles, respectively) and previous studies (i.e. using two-dimensional difference gel electrophoresis, 2D-DIGE to investigate changes in protein expression profile). Other cold shock proteins that were significantly up-regulated upon cold shock included *cspCH* (at time 330 min). This is consistent with the fact that CspC has been reported to be constitutively expressed at both high and low temperature. CpsC appears to be implicated in chromosome condensation and cell division (Phadtare and Inouye 2008). Furthermore, other groups of genes and proteins previously shown to be cold-induced were found to be significantly up-regulated at all time points, including an ATP-dependent helicase (*csdA*/CsdA) (Jones et al. 1996) and exoribonuclease R (*rnr*/Rnr) (Cairrao et al. 2003). The observed increase in expression of these genes and proteins throughout exposure to cold stress suggests their importance beyond the initial phase of the acclimation period.

Genes and proteins of the RpoS regulon (Lacour and Landini 2004; Patten et al. 2004; Weber et al. 2005) were up-regulated with significant *T*-value from 90 min of the cold stress onward (Fig. 3.10). This is consistent with previous reports of low temperature induction of RpoS (White-Ziegler et al. 2008; Kocharunchitt et al. 2011). A total of 19 genes exhibited a significant

increase in expression from 90 min onward and included genes involved in the osmotic stress response (*osmY*), acid stress response (*gadX*), biofilm formation (*bolA*), cell wall biogenesis (*erfK*), encoding an inner membrane protein (*yqjE*), predicted outer membrane protein (*ybaY*), hypothetical proteins (*yahO*, *yaiA*, *yccJ*, *ydiZ*, *yodC*, *yqjCD*, *yhhA*, *ytfK* and *ygaM*) and putative and predicted transcriptional regulators (*yiaG*, *yjbJ*, *nsrR*). In contrast, abundance of only two proteins of the RpoS regulon was found to be significantly induced from 90 min onward including an osmotic stress response protein (OsmY) and a putative outer membrane protein (YeaY).

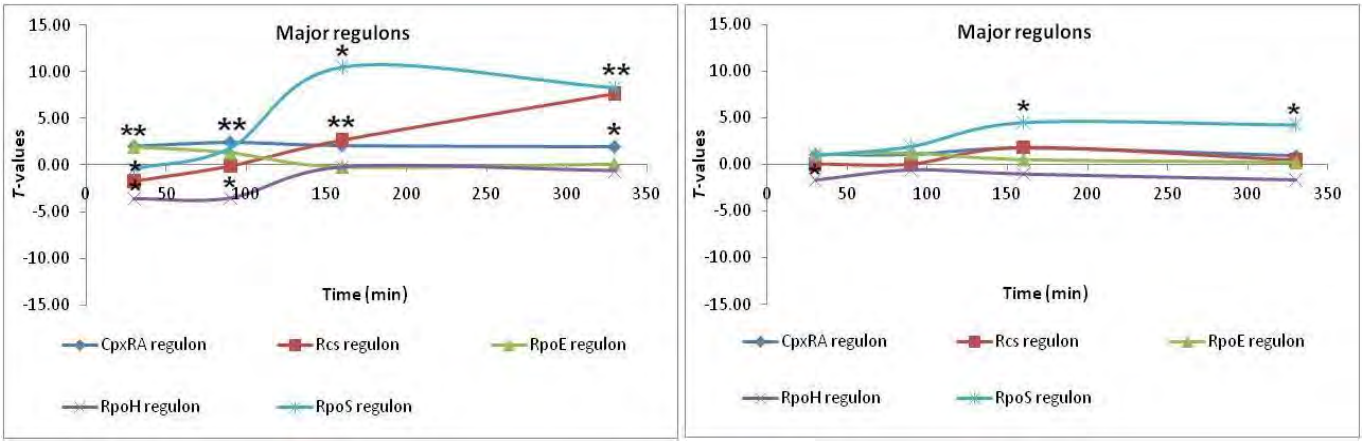

Fig. 3.10. Changes in overall expression of genes (left panel) and proteins (right panel) involved in the major regulons during exposure of *E. coli* O157:H7 Sakai to temperature downshift from 35°C to 14°C, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the list of genes or proteins previously known to be induced by major regulons, as described in Section 3.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

The RpoS-dependent *otsAB* genes were also up-regulated by up to 2.61 $\log_2$-fold from 160 min of the cold stress onward (Appendix E.3). This is in keeping with the induction of *otsAB* and activation of trehalose synthesis during cold shock reported by Kandror et al. (2002) and Moen et

al. (2009). Kocharunchitt et al. (2011) has also revealed an increase in the expression of trehalose biosynthetic enzymes (*otsAB*/OtsAB) under the stress condition of 14°C a_w 0.985. Trehalose is thought to enhance cell viability when the temperature is reduced to near freezing (Kandror et al. 2002; Phadtare 2004).

The RpoE extracytoplasmic stress response regulon was initially up-regulated, as indicated by a significant positive *T*-value in the transcriptomic data at 30 min of the stress (Fig. 3.10). This was despite the observation that the *rpoE* gene encoding the RpoE sigma factor was significantly up-regulated by up to 2.13 $\log_2$-fold within the first 90 min. A total of 16 genes within the RpoE regulon (Rhodius et al. 2006) exhibited up-regulation at time 30 min including *rpoE* and its regulators (*rseABC*); the nitrogen-related (*rpoN*) and heat shock (*rpoH*) sigma factors; genes associated with lipid A biosynthesis (*ddg*), zinc export (*zntR*), asparagine metabolism (*ansB*) and phosphopantetheinyl transfer (*yieE*); a stress-inducible periplasmic protease (*degP*); a helicase (*lhr*); regulators involved with pyrimidine metabolism, purine degradation and pH homeostasis (*rutR*); fatty acid biosynthesis (*fabR*); as well as genes encoding an outer membrane protein (*yidQ*), and a predicted protein (*yfeK*). In the proteomic analysis, putative outer membrane lipoprotein of the RpoE regulon (YeaY) was found to be amongst the most highly up-regulated proteins at all time points. Consistent with these findings, Polissi et al. (2003) reported that *rpoE* exhibited an early transient increase in mRNA abundance 1 h after cold shock at 16°C. RpoE is thought to be activated by, and respond to, misfolded proteins in either the periplasm or outer membrane (Raivio and Silhavy 1999). However, it remains to be determined how the RpoE regulatory pathway is involved in low temperature adaptation in response to cold induced extracytoplasmic stress (Polissi et al. 2003).

Expression of the *rpoH* gene was induced (up to 2.55 $\log_2$-fold) throughout the course of cold stress. However, related to the RpoH regulon (Nonaka et al. 2006), genes (within the first 90 min after the shift) and proteins (at time 30 min) were down-regulated (Fig. 3.10). Within the first 90 min of the stress, seven genes encoding heat shock chaperones (*dnaKJ*, *htpG*, *ppiB*, *grpE* and *groEL*) were commonly down-regulated. Genes and proteins of the RpoH regulon, which were commonly down-regulated included *htpG* (at time 30 and 330 min) and HtpG (at time 330 min), and *prlC* (at time 90 min) and PrlC (at time 330 min). This is not surprising in light of the role of HtpG as a heat shock chaperone and the fact that PrlC is believed to be involved in the onset of DNA replication (Kato et al. 1992), for which a general down-regulation was observed in the proteomic data (at time 30, 160 and 330 min; Appendix E.3). The observed down-regulation of chaperones may also be a contributing factor to the growth arrest of *E. coli* following cold shock. Indeed, it has previously been shown that the growth arrest of *E. coli* upon cold shock appeared to be associated with inactivation of the chaperone system (Strocchi et al. 2006).

The T-profiler analysis revealed a significant increase in overall expression of genes previously known to be positively controlled by the CpxRA regulon, which is involved in the envelope stress response and in the control of the biogenesis of adhesive organelles such as pili (Price and Raivio 2009) (Fig. 3.10). However, this was not evident in the proteomic data. Genes of the CpxRA regulon that were significantly up-regulated at all time points included those involved in spheroblast formation (*spy*), regulation of the extracytoplasmic stress response (*yihE*) and murein biosynthesis (*ycfS*). Both genes encoding the Cpx system also exhibited a significant increase in expression throughout the time course (*cpxR*; at time 90 and 160 min, *cpxA*; at time 160 and 330 min). Other evidences of envelope stress were indicated by a significant induction of *degP*,

encoding a stress-inducible periplasmic protease (within the first 160 min after temperature downshift). In line with the down-regulation of chemotaxis and motility genes observed here (Section 3.3.2.7), *ydeH* was up-regulated by up to 2.50 $\log_2$-fold from 90 min of the shift onward. The *ydeH* gene encodes a diguanylate cyclase typically regulates motility and biofilm formation and represses swimming behavior by reducing the abundance of flagella and abolishes the appearance of pili-like structures (Jonas et al. 2008).

Activation of the Rcs regulon (Hagiwara et al. 2003), which controls the biosynthesis of extracellular polysaccharide capsule or colanic acid was observed in the transcriptomic study from 160 min of the shift onward (Fig. 3.10). However, a number of genes and proteins involved in colanic acid biosynthesis exhibited a significant up-regulation at 30 (*ugd*), 160 (*ugd*, *cpsB*, *fcl*, *gmd*) and 330 (GalK) min. Genes and proteins known to be induced by the Rcs regulon were amongst the most highly up-regulated under all steady-state conditions (Kocharunchitt et al. 2011). The present findings are also consistent with the report of Hagiwara et al. (2003) concerning induction at low temperatures.

A number of genes within the acid fitness island (AFI) were found to be significantly up-regulated at 30 (*hdeD* and *gadEX*), 90 (*hdeD* and *gadEX*), 160 (*hdeBD*, *gadEX, dctR* and *yhiD*) and 330 (*dctR*, *hdeB* and *gadEX*) min of the cold stress. In contrast, the proteomic analysis only revealed a significant up-regulation of starvation lipoprotein (Slp) at 90 and 330 min (4.21 $\log_2$-fold on average). This supports earlier suggestion that growth at low temperature may result in energy deficiency. The apparent up-regulation of glutamate decarboxylase genes (i.e. *gad* operon) during exposure to cold stress also agrees well with the observations of Kocharunchitt et

al. (2011) and Moen et al. (2009). Polyamines (e.g. putrescine and spermidine) are known to play an important role during acid stress (Yohannes et al. 2005), and in the present study the transcriptomic data revealed up-regulation of elements of the *opp* operon (*oppAC*) that encodes a polyamine-induced oligopeptide ABC transport system at 90 min and 160 min.

Interestingly, several genes involved in response to oxidative damage exhibited a significant down-regulation at 30 (*dps*, *grxA*, *cysK*, *ibpA* amd *katG*), 90 (*yaaA*, *ahpF*, *dps*, *grxA*, *sufABC*, *yeeD*, *cysK*, *ibpA* and *katG*), 160 (*ahpF*, *grxA*, *sufC*, *yeeD*, *ibpA* and *katG*) and 330 (*grxA*, *sufC*, *ibpA* and *katG*) min of the cold stress. This was despite the observation that abundance of KatG was reduced from 90 min of the stress onward. These elements are reported to be amongst the most highly up-regulated during exposure to hydrogen peroxide (Zheng et al. 2001). Mackey and Derrick (1986) have also demonstrated that *E. coli* become sensitive to oxidative stress upon cold shock.

3.4 CONCLUDING REMARKS

This study offers a detailed understanding of the growth kinetics and molecular response of exponential phase *E. coli* O157:H7 Sakai to an abrupt temperature downshift. Shifting cells from 35°C to a lower temperature (i.e. 20°C, 17°C, 14°C or 10°C) resulted in a lag period (i.e. no-growth) before growth resumed at a rate typical of the new temperature. Both lag and generation times appeared to increase with respect to the magnitude of the shift. Of particular interest, the RLT response of *E. coli* to cold shock displayed little variation across the tested temperature range, indicating that *E. coli* undertakes a similar amount of *work* to adapt to each of these temperatures.

Assessment of the global transcriptomic and proteomic response of *E. coli* O157:H7 Sakai to an abrupt temperature downshift from 35°C to 14°C revealed a general decrease in the global expression of elements involved in DNA replication, transcription, translation and protein synthesis. This response parallels the observed decrease in growth rate of *E. coli* upon temperature downshift, and presumably also the necessity for saving and reallocating energy for repairing damage to the cell. Numerous changes occurred in the expression pattern of genes and proteins involved in transport and binding functions to contend with the effects of cold temperature on the bacterial cell envelope. The selective induction of specific amino acid biosynthetic pathways involved in histidine and the aromatic amino acid biosynthetic families also suggests a role for these amino acids in adaptation to; and growth under, chill temperature. The expression pattern of the global stress response regulons provided insight into their roles, with sustained expression of the RpoS-dependent general stress response pathway and an initial transient induction in the RpoE-dependent extracytoplasmic stress response pathway at 30 min when cultures were in the lag phase and adapting to the conditions of growth. The results of this study highlight those alterations in gene expression and protein abundance that are required for adaptation to, and growth under, conditions of chill temperature stress, and will help to understand the physiological response of *E. coli* to simultaneous abrupt downshifts in temperature and water activity stress (Chapter 5).

CHAPTER 4

GLOBAL GENOMIC AND PROTEOMIC RESPONSES OF *ESCHERICHIA COLI* O157:H7 SAKAI DURING DYNAMIC CHANGES IN GROWTH KINETICS INDUCED BY AN ABRUPT DOWNSHIFT IN WATER ACTIVITY

4.1 INTRODUCTION

Increasing the osmotic pressure (lowering water activity, a_w) is one of the most widely used methods in food preservation to control the growth of bacteria, including *E. coli.* Reduction in the external a_w typically results in a rapid loss of the cytoplasmic volume in a process called plasmolysis, and causes reduced respiration and growth arrest, whereas both intracellular ATP and cytoplasmic pH have been reported to increase (Csonka 1989; Weber et al. 2006). To adapt to hyperosmotic stress, bacteria employ adaptive mechanisms referred to generally as osmoregulatory systems. A major role of these systems is to maintain the intracellular osmotic pressure within tolerable limits. This generally involves accumulation of charged solutes (e.g. potassium ions (K^+) and glutamate), followed by accumulation of compatible solutes either through *de novo* biosynthesis (e.g. trehalose) or through uptake from the external environment (e.g. glycine betaine and proline) (O'Byrne and Booth 2002; Chung et al. 2006). Furthermore, it is well established that bacterial cells previously exposed to osmotic stress (i.e. osmotically-adapted cells) acquire increased resistance to other stresses such as high temperature and oxidative stresses (Chung et al. 2006; Gunasekera et al. 2008). Therefore, the ability of pathogenic bacteria to adapt to and survive under adverse conditions could increase the risk of food-borne illness. A detailed understanding on how *E. coli* O157:H7 adapts to hyperosmotic

shift is particularly important in food processes where prevention of bacterial growth or elimination of contamination is critical.

Previously, both cDNA microarray and 2D-LC/MS/MS analyses were employed to elucidate the genome and proteome profiles of exponential phase *E. coli* O157:H7 strain Sakai grown under steady-state conditions, relevant to low temperature and water activity conditions experienced during carcass chilling (Chapter 2; Kocharunchitt et al. 2011). It was found that *E. coli* O157:H7 respond to these steady-state conditions, including osmotic stress (25°C a_w 0.967) by activating the master stress response regulator RpoS and the Rcs phosphorelay system involved in the biosynthesis of the exopolysacharide colanic acid, as well as down-regulating genes and proteins involved in chemotaxis and motility (Kocharunchitt et al. 2011). Such findings have provided a baseline of knowledge of the potential molecular mechanisms enabling growth and survival of this pathogen under these stress conditions. To gain a deeper insight into the physiology of exponentially growing *E. coli* O157:H7 Sakai in response to hyperosmolality, the present study was undertaken to investigate the growth kinetics of this pathogen subjected to sudden osmotic upshift (from a_w 0.993 to a_w 0.980, 0.975, 0.970, 0.967 or 0.960), as well as to examine the time-dependent alterations in its genome and proteome upon hyperosmotic shock from a_w 0.993 to a_w 0.967 at a constant temperature of 35°C. The global responses were analyzed by both cDNA microarray and 2D-LC/MS/MS analyses. Differences in gene expression and protein abundance patterns in *E. coli* before and after osmotic shock were analyzed through quantitative and comparative analysis of time-series changes in both mRNA and protein levels.

4.2 MATERIALS AND METHODS

4.2.1 Division of experimental work

A series of experiments on the growth response of *E. coli* to hyperomotic shift were conducted in the Food Safety Centre, University of Tasmania (Hobart, Australia). Parallel transcriptomic and proteomic studies were, however, undertaken in two independent laboratories, CSIRO Food and Nutritional Sciences (North Ryde, Australia) and the Food Safety Centre (Hobart, Australia), respectively. The differences in the protocol followed at each site are described in Section 2.2.1.

4.2.2 Bacterial strain

All experiments described in the present study employed *E. coli* O157:H7 stain Sakai (Section 2.2.2).

4.2.3 Preparation of low a_w broth

A brain-heart infusion (BHI) broth (CM225, Oxoid, Australia; Appendix A.7) containing a very high concentration of NaCl was prepared to be used in osmotic upshift experiments. An appropriate amount of NaCl was added to fresh BHI broth to achieve the final a_w of 0.760 (± 0.003), as determined from the Tables of Chirife and Resnik (1984). This NaCl-concentrated BHI broth was then sterilized (121°C, 20 min) and kept at room temperature until used. The final a_w of the NaCl-concentrated BHI broth was confirmed using an Aqualab CX-2 dew point instrument (Decagon Devices, Inc., Pullman, USA).

4.2.4 Abrupt downshift in water activity

A stationary phase culture of *E. coli* (Section 2.2.3) was diluted 1:10^4 in 25 ml of pre-warmed (35°C) BHI broth. This 'primary' culture was incubated at 35°C with agitation (80 oscillations.min^{-1}) in a water bath (Ratek Instruments, Australia), and its growth monitored turbidimetrically at 600 nm with a Spectronic 20 spectrophotometer (Bausch and Lomb, USA). After achieving an optical density at 600 nm (OD_{600}) of 0.1 ± 0.01 (i.e. the mid-exponential phase of growth, ~10^7 CFU.ml^{-1}), the primary culture was diluted 1:10^2 in a fresh 25 ml of pre-warmed (35°C) BHI broth to prepare a 'secondary' culture. This culture was then incubated at 35°C with agitation until it reached the mid-exponential growth phase (OD_{600} of 0.1 ± 0.01). At this point, an appropriate volume of the NaCl-concentrated BHI previously prepared (pre-warmed to 35°C) was added to the culture to create a series of hyperosmotic broths at a_w 0.980, 0.975, 0.970, 0.967 or 0.960. The test broth at each a_w was incubated at 35°C with shaking.

4.2.5 Microbiological analysis

E. coli growth was determined by viable count method. At regular intervals, 0.1 ml of the test broth was obtained and serially diluted in 0.1% peptone water (PW; LP0037, Oxoid, Australia; Appendix A.9) containing 0.85% NaCl as required. Aliquots of the appropriate dilution were then plated onto BHI agar supplemented with 0.1% sodium pyruvate (P8574, Sigma, USA; Appendix A.6) using an Autoplate 4000 spiral plater (Spiral Biotech, Bethesda, USA). All plates were then incubated at 37°C for 24 h before colonies were counted. All growth experiments were replicated at least twice, and the results are reported as the average of the replicates.

Log_{10} CFU.ml^{-1} was plotted against time to generate growth curve for each osmotic treatment. All growth curves were analyzed using linear regression to estimate generation time (GT) and lag time (LT) according to Section 3.2.4. The present study also determined the time taken for cells to resume the exponential growth following the shift. This is referred to as 'adaptation time' (AT) and was estimated visually from the growth curves. To measure the relative amount of *work* to be done by *E. coli* to adjust to new conditions, relative lag time (RLT) was calculated by dividing LT or AT by GT (Robinson et al. 1998; Mellefont and Ross 2003).

4.2.6 Transcriptomic and proteomic analysis

In a separate set of hyperosmotic shift experiments, a rapid downshift of *E. coli* cultures (five lots in total) from a_w 0.993 to a_w 0.967 was carried out as described above (Section 4.2.4) to prepare samples for cDNA microarray and 2D-LC/MS/MS analysis. Each lot of the cell cultures (25 ml) was, respectively, harvested before osmotic upshift (i.e. non osmotically-treated cells), and at 0 (i.e. immediately after the shift), 30, 80, and 310 min after the shift. Independent samples were then subjected to extraction of RNA, and both soluble and membrane proteins according to Section 3.2.5. The number of biological replicates performed for each time point in the transcriptomic and proteomic analysis is shown in Table 4.1.

Table 4.1 Number of biological replicates performed in cDNA microarray and 2D-LC/MS/MS analysis.

Time points	Number of biological replicates	
	Transcriptome	Proteome[a]
Before a_w downshift	3	6(6)
Immediately after shift	3	2(2)
30 min after shift	3	2(2)
80 min after shift	3	2(2)
310 min after shift	3	2(2)

[a] Number of replicates performed for soluble (outside brackets) and membrane (within brackets) fractions of *E. coli*.

Analysis of cDNA microarray and 2D-LC/MS/MS was respectively performed on RNA and proteins as described in Sections 2.2.8 and 2.2.12.

4.2.7 Microarray data analysis

Microarray data were subjected to background correction, normalization and calculation of the expression measures according to Section 2.2.9. Significance analysis was conducted using one way analysis of variance (ANOVA) using the multiple correction testing method of Benjamini and Hochberg (1995) with a *P*-value cut-off of <0.01. Elements were considered to be differentially expressed if the fold change was >2.

4.2.8 MS/MS data analysis

MS/MS spectra obtained were processed and searched against the complete database of *E. coli* O157:H7 Sakai (5,318 entries in total) as described in Section 2.2.13. All protein identifications that passed the criteria (i.e. a PeptideProphet and ProteinProphet of ≥0.9) were then assigned a confidence level as described in Section 3.2.7. Only protein identifications with a 'high' and 'intermediate' confidence level (referred as having a 'high confidence score') were accepted for further analysis.

Following data filtering, all protein identifications with high confidence from membrane and soluble fractions of the same sample were combined to represent a 'total' fraction of the corresponding sample. This combined approach was carried out to improve reproducibility of the proteomic data as described in Section 3.2.7. The R^2 values, as determined by linear regression analysis, indicated a relatively stronger linear correlation between spectral counts (SpCs, the

number of MS/MS spectra) for all possible pair-wise comparison of replicates of pooled fraction (average $R^2 = 0.89 \pm 0.06$) when compared to that of replicates of each fraction (average $R^2 = 0.64 \pm 0.20$, and 0.85 ± 0.06 for membrane and soluble fractions, respectively). Each of the total fractions across the biological replicates was then used to generate the final list of proteins identified at each time point of sampling.

4.2.9 Protein abundance ratio and its significance

Spectral count generated by 2D-LC/MS/MS analysis was used as a semi-quantitative measure of protein abundance (Nesvizhskii et al. 2003; Liu et al. 2004). The normalized spectral abundance factor (NSAF) for each protein was calculated as detailed in Section 3.2.8.

Fold changes in protein abundance due to hyperosmotic shock were calculated as described in Section 3.2.8. Significance analysis was carried out using the beta-binomial test implemented in R (Pham et al. 2010). All proteins with a *P*-value <0.01 and at least 2-fold change were considered to be differentially expressed.

4.2.10 Transcriptomic and proteomic data mining

Information on identified genes and proteins including protein and gene names, ECs numbers (locus tag), GI numbers, NCBI Reference Sequence (RefSeq), protein sizes and molecular masses, as well as their preliminary functions and properties were obtained from public databases as described previously (Section 3.2.9). In the present study, protein or gene names in conjunction with ECs numbers were used as a unique identifier for identified genes and/or proteins.

4.2.11 Predefined set enrichment analysis

To determine changes in overall expression of a predefined set of genes or proteins due to osmotic upshift, the T-profiler analysis was performed on the $\log_2$ ratios according to Section 2.2.16. The predefined sets of genes or proteins used for the present analysis were based on the selected functional role categories and/or metabolic pathways of the databases as given in Section 3.2.10. The *T*-value obtained from the analysis was determined only for sets that contained at least five members, and its significance was established by using the associated two-tailed *P*-value. All predefined sets with a *P*-value less than 0.1 were considered to be statistically significant.

4.3 RESULTS AND DISCUSSION

4.3.1 Growth of *E. coli* O157:H7 Sakai upon a_w downshift

The growth kinetics of *E. coli* was assessed during exponential growth (OD_{600} of ~0.1 ± 0.01) in a nutrient rich medium when subjected to a rapid downshift from a_w 0.993 to either a_w 0.980, 0.975, 0.970, 0.967 or 0.960 at a constant temperature of 35°C. All downshifts resulted in a growth interruption before cells resumed their growth at a slower rate (Fig. 4.1). Estimates of kinetic parameters for each a_w downshift are summarized in Table 4.2. The generation times obtained were consistent with those expected from the predictive model of Ross et al. (2003), and both generation and lag times increased with respect to the magnitude of the osmotic shift. The $RLT_{LT/GT}$ response of *E. coli* also showed to increase in a linear manner ($y = -225.64x - 1.84$, $R^2 = 0.89$) with decreasing a_w tested (Fig. 4.2). This is consistent with the previous studies, reporting that shifting cells to progressively harsher conditions would require more adaptation *work* to be done, i.e. RLTs become larger (Robinson et al. 2001; Mellefont et al. 2003, 2005).

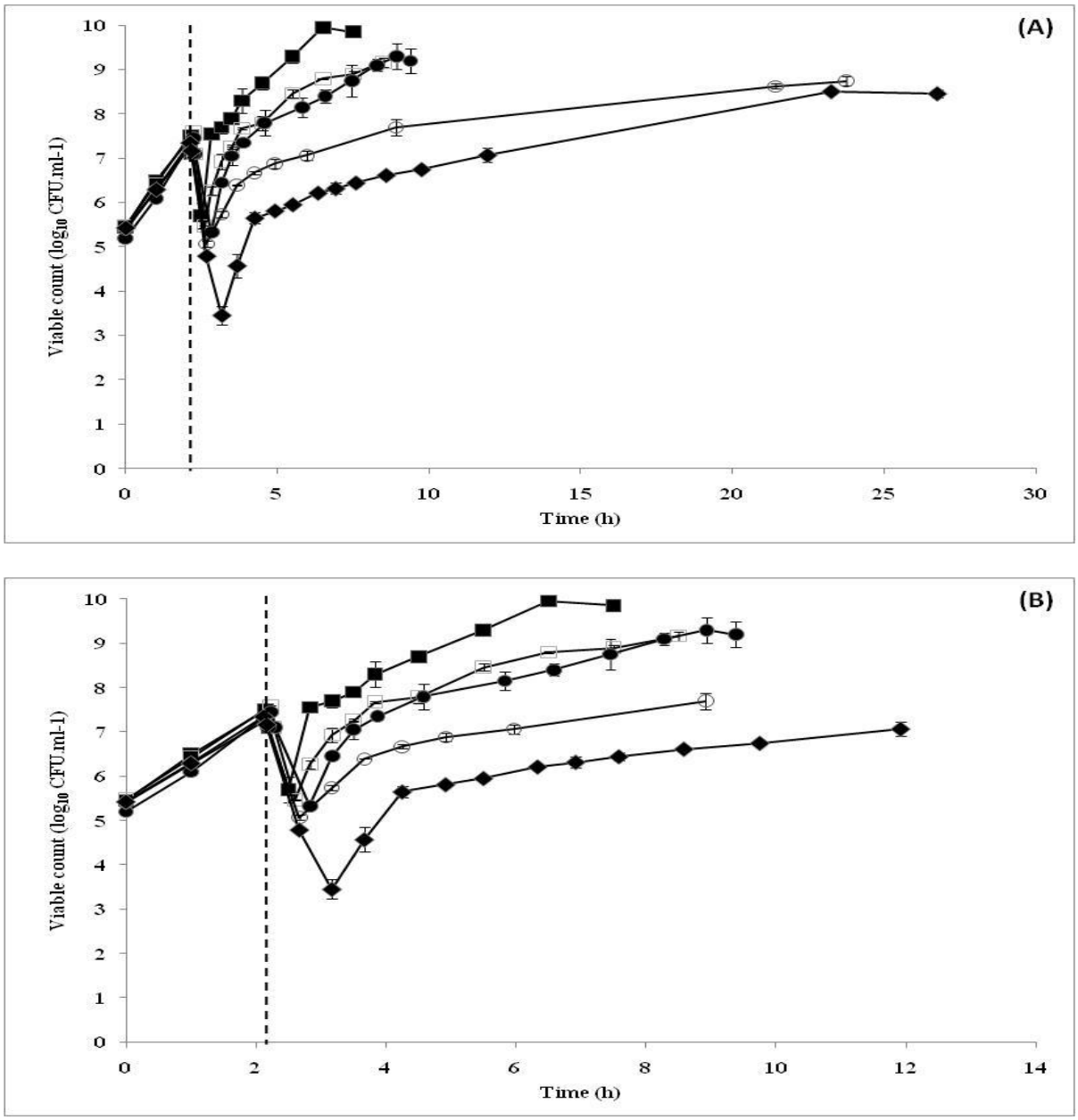

Fig. 4.1. Growth of *E. coli* O157:H7 Sakai in response to a sudden downshift in water activity from a_w 0.993 to a_w 0.980 (■), 0.975 (□), 0.970 (●), 0.967 (○) and 0.960 (◆) at a constant temperature of 35°C, as determined by viable count. The time at which hyperosmotic shift was applied is indicated by a dotted line. Data points represent means ± standard deviations of at least two independent replicates. Panel (A) shows all available data and panel (B) shows all data on a reduced time scale.

Table 4.2 Summary of the growth response of exponential phase *E. coli* O157:H7 Sakai to a sudden downshift in water activity from a_w 0.993 to a_w 0.980, 0.975, 0.970, 0.967 and 0.960, as determined by viable count.

Water activity downshift	Generation time, GT (h)[a]	Lag time, LT (h)[b]	Adaptation time, AT (h)[c]	Relative lag time (RLT)	
				LT/GT	AT/GT
a_w 0.980	0.45	0.69	0.68	1.53	1.51
a_w 0.975	0.67	1.55	0.94	2.31	1.40
a_w 0.970	0.73	2.04	1.27	2.79	1.74
a_w 0.967	1.41	4.74	1.53	3.36	1.09
a_w 0.960	1.68	10.57	2.13	6.29	1.27

[a] Generation time (GT) is determined by linear regression

[b] Lag time (LT) is calculated by linear regression as the time taken to increase above starting numbers.

[c] Adaptation time (AT) is estimated visually as the time taken for cells to resume the exponential growth.

E. coli appeared to have a complex pattern of growth behavior in response to a sudden downshift in a_w. A rapid decrease in bacterial numbers was observed immediately after a shift to low a_w (Fig. 4.1). This indicates that cells might lose their culturability on enumeration media, possibly due to injury. The degree of loss of culturability also became progressively larger with the larger shifts in a_w, although this loss of culturability occurred at a similar rate for all a_w tested. However, the loss of culturability was reversed within the lag period at a rate faster than that characteristic of exponential growth under the given stress condition (Fig. 4.1). Furthermore, it was evident that during treatment at a_w 0.967 and 0.960, the exponential growth occurred before the lag period (if defined as the time taken to increase above starting numbers) was resolved. This suggests that cell death might occur upon the shift at the two lowest a_w tested. A similar observation of injury and recovery phenomenon has been reported in other studies following the application of sub-lethal stressful treatments (Busta 1978; Mackey and Derrick 1982; Zhou et al. 2010). However, these results are in contrast to the study of Mellefont et al. (2005) in which the abrupt osmotic shifts of *Salmonella* Typhimurium caused an inactivation (rather than loss of culturability) of a sub-population followed by renewed exponential growth.

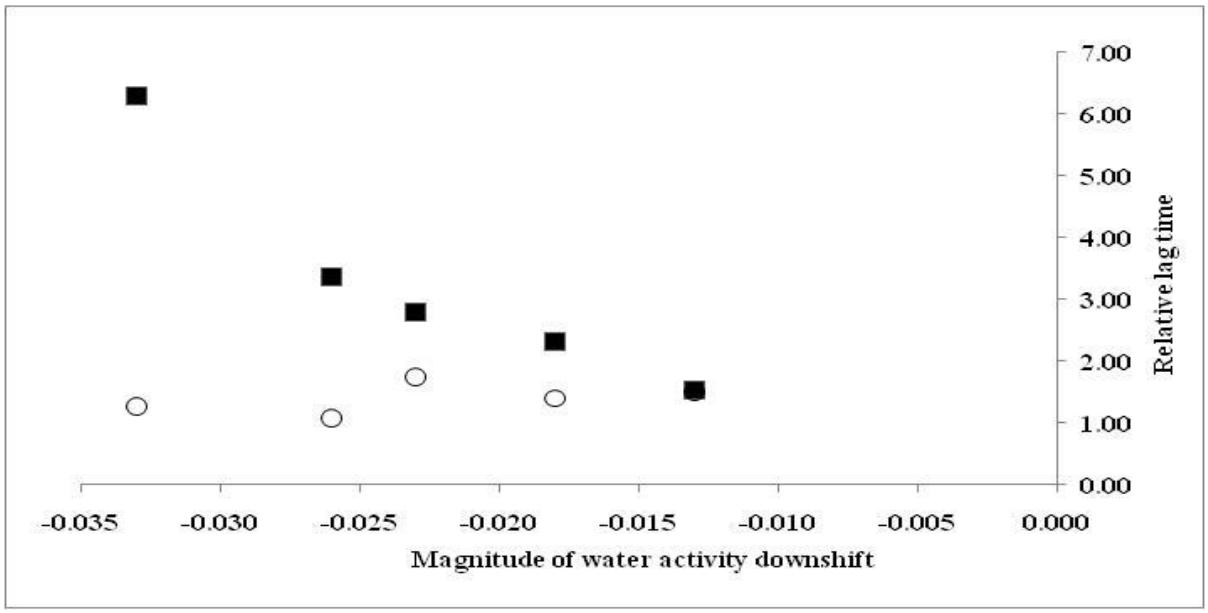

Fig. 4.2. Effects of a rapid downshift in water activity from a_w 0.993 to a range of low a_w on the relative lag time response of exponential phase *E. coli* O157:H7 Sakai at a constant temperature of 35°C. RLT estimate was calculated by dividing lag time **(■)** or adaptation time **(○)** with generation time.

The complexity of the response of *E. coli* to osmotic upshifts (i.e. irregularly shaped growth curves arising from the initial decline and subsequent recovery in cell numbers) may contribute to unusual estimates of RLT, although a consistent and reproducible pattern of the $RLT_{LT/GT}$ response was obtained in this study. Injured or dead cells may mask the time when the lag period is actually resolved, resulting in overestimation of lag time. This has led the candidate to determine adaptation time (AT), which refers to the time taken for bacterial cells to resume exponential growth after hyperosmotic shift. The adaptation times increased with the magnitude of the osmotic shift, a similar trend to that observed for lag times (Table 4.2). However, when RLT estimates were calculated using the adaptation time, there was no systematic variation in $RLTs_{AT/GT}$ over the a_w range tested ($y = 14.53x + 1.73$, $R^2 = 0.20$; Fig. 4.2). This implies that *E. coli* cells require a similar amount of *work* to be done to adapt to a given decrease in a_w and,

thus, is inconsistent with the observed pattern of $RLT_{LT/GT}$ response and previous studies in which shifts to progressively harsher environments would require more adaptation *work* to be done (Robinson et al. 2001; Mellefont et al. 2003, 2005). Further investigations at greater downshifts together with validation of the data by culture-independent methods, are necessary to confirm the present observations.

4.3.2 Molecular response of *E. coli* O157:H7 Sakai to hyperosmotic shock

To understand better the regulatory network involved in osmotic adaptation, both transcriptomic and proteomic analyses were employed in parallel to characterize temporal alterations in mRNA expression and protein production in *E. coli* upon a rapid downshift from a_w 0.993 to a_w 0.967 at a constant temperature of 35°C (Fig. 4.1). Bacterial samples were taken before hyperosmotic shift, and 0 (i.e. immediately after the shift), 30, 80, and 310 min after the shift (Fig. 4.3). It also should be noted that the samples taken at time 0 and 30 min were obtained during the period in which the loss of cell culturability was observed, whereas the samples at time 80 and 310 min respectively reflected the physiological state of *E. coli* during the 'recovery' period and growth after the shift. Independent samples were processed for mRNA, and membrane and soluble protein extractions as appropriate for analysis of cDNA microarray and 2D-LC/MS/MS, respectively. By using multidimensional LC/MS/MS analysis together with data filtering, protein identifications with high confidence were detected in total fractions of *E. coli* at each time point (Appendix F.1 and Table 4.3). It is noteworthy that a minority of these proteins were uniquely identified from the membrane fraction, ranging from 6.9% to 23.1% of protein identifications across all proteomic datasets. This implies that additional extraction of membrane fractions provided a slight improvement in proteome coverage. In addition, all datasets were analyzed for

false-positive discovery rate of peptides by dividing the number of spectra matching decoy peptides with the total number of spectra. The false-positive rate of peptide identifications was less than 5% for all datasets (Appendix F.2).

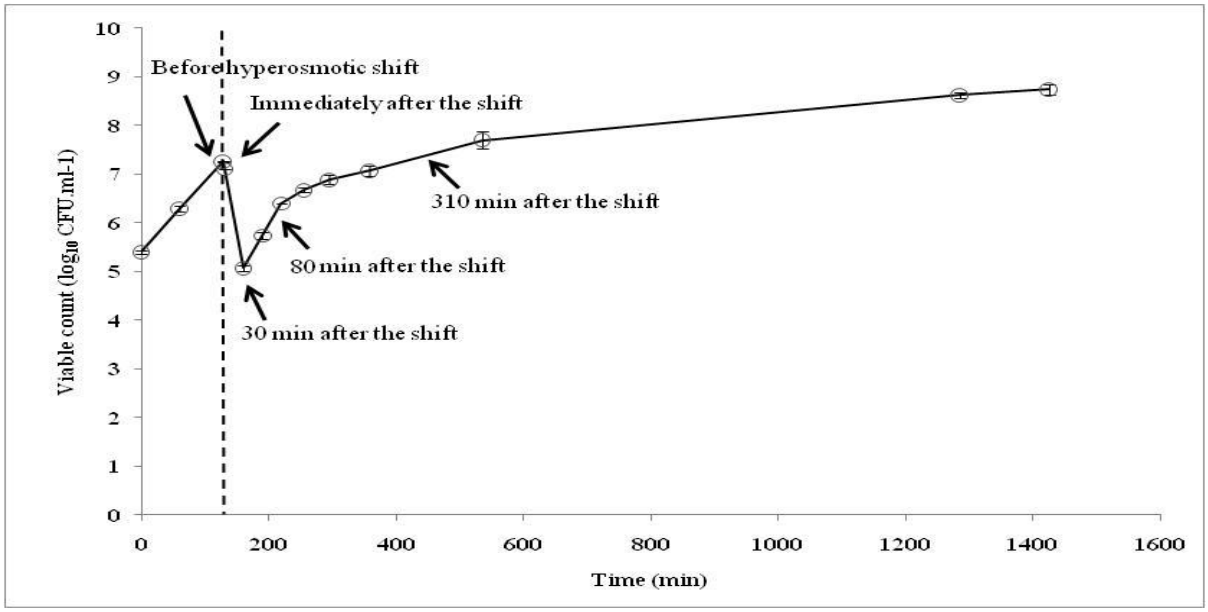

Fig. 4.3. Sampling scheme for analysis of molecular response of *E. coli* O157:H7 Sakai to hyperosmotic shift from a_w 0.993 to a_w 0.967. The time at which hyperosmotic shift was applied is indicated by a dotted line. Block solid arrows indicate the sampling point for cDNA microarray and 2D-LC/MS/MS analyses. Data points represent means ± standard deviations of at least two independent replicates.

Table 4.3 The number of protein identifications and differentially expressed genes and proteins during exposure of *E. coli* O157:H7 Sakai to hyperosmotic stress.

Time points	Number of proteins			Number of differentially expressed elements[d]		
	Membrane fraction[a]	Soluble fraction[a]	Total fraction[b]	Trancriptome (total)[e]	Proteome (total)	Transcriptome *vs.* Proteome
Before a_w downshift	343	1,281	1,225(61)[c]	NA[f]	NA	NA
Immediately after shift	710	865	1,020(233)	3(5)	158(30)	0(0)
30 min after shift	649	901	1,064(246)	182(15)	162(21)	2(0)
80 min after shift	615	904	968(218)	772(414)	126(37)	16(8)
310 min after shift	1,071	1,259	1,416(252)	605(326)	399(12)	145(9)

[a] Number of proteins identified in membrane and soluble fractions of the *E. coli* proteome that pass the filtering criteria (i.e. at PeptideProphet and ProteinProphet of ≥0.9).

[b] Number of protein identifications with high confidence in total fraction of the *E. coli* proteome (outside brackets).

[c] Number of high-confidence proteins uniquely identified in membrane fraction (within brackets).

[d] Number of elements with increased (outside brackets) and decreased (within brackets) expression.

[e] Analysis does not include the number of differentially expressed undefined intergenic regions in the transcriptome.

[f] NA; not applicable.

Changes in gene expression and protein abundance due to hyperosmotic stress were determined by comparing the profiles obtained after the stress to that of a reference sample taken prior to the stress. Table 4.3 describes the number of genes and proteins with significant changes in expression level at each time point. It was found that the number of up-regulated elements was dominant over those with decreased expression level. A complete list of these genes and proteins with annotations, as well as their comparison to previously published studies is shown in the Appendix F.3. Furthermore, the transcriptomic and proteomic profiles at each time point were compared. The level of correlation, as determined by calculating the percentage of differentially expressed genes and proteins matched was found to be low with an average of only 4.32% ± 7.09% of elements matched across all time points (see Appendix F.4), although a number of trends were still observed in the data (see below).

Exposure of *E. coli* to a rapid downshift in a_w evokes a highly complex regulatory process involving expression changes of different groups of genes and proteins, which here was condensed using the T-profiler analysis (For full results see Appendix F.5). These genes and proteins are involved in a number of functional categories and metabolic pathways, as well as play an important role in the stress responses that are controlled by regulons, as discussed below.

4.3.2.1 Transcription and translation

Bacteria generally control the cellular content of transcriptional and translational machineries according to their physiological state. Faster-growing cells synthesize proteins more quickly, and the level of translation apparatus proteins correlates well with growth rate (Gyaneshwar et al. 2005; Klumpp et al. 2009). Reduced growth rate as a result of environmental conditions including high osmolarity has an effect on gene expression and protein production (Weber and Jung 2002; Kocharunchitt et al. 2011). However, the present findings did not show a strong alteration in the expression of genes and proteins associated with RNA polymerase in response to a_w downshift (Fig. 4.4). This was despite the transcriptomic analysis revealing a significant down-regulation of *pcnB* encoding poly(A) polymerase I (1.06 $\log_2$-fold) only at 80 min of the treatment. On the other hand, it was found that the amount of translational machinery was affected after prolonged exposure to osmotic stress. Several ribosomal proteins exhibited a decrease in their abundance at 310 min after the treatment, as indicated by a significant negative *T*-value of 4.39 (Fig. 4.4). This result agrees well with the observations of Kocharunchitt et al. (2011), reporting a reduced capacity of translation in osmotically-adapted cells.

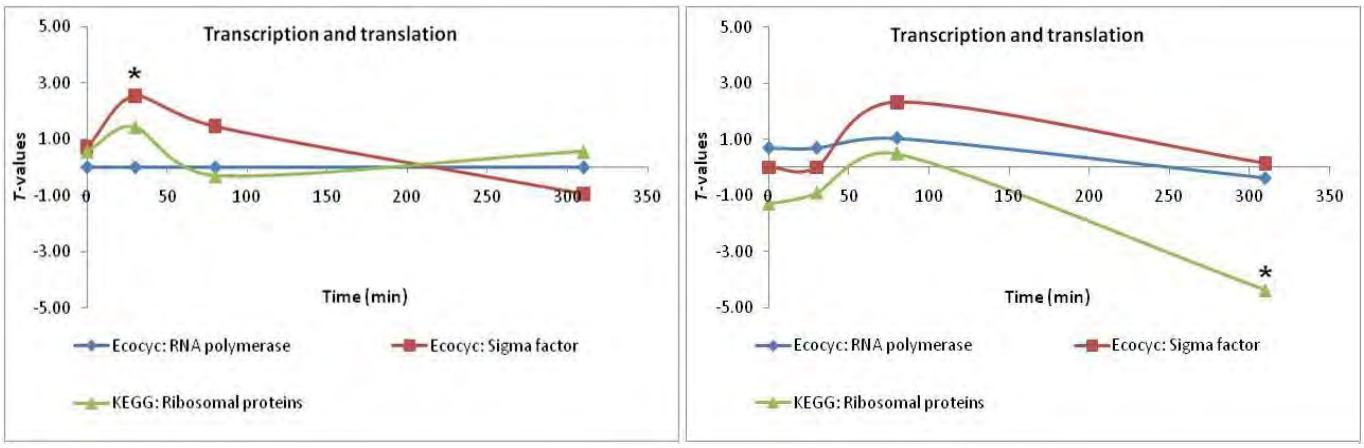

Fig. 4.4. Changes in overall expression of genes (left panel) and proteins (right panel) involved in transcription and translation during exposure of *E. coli* O157:H7 Sakai to hyperosmotic shift from a_w 0.993 to a_w 0.967, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated databases, as described in Section 4.2.11. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

4.3.2.2 Carbohydrate catabolism and energy generation

A sudden downshift in a_w was generally found to have no major effects on expression of genes and proteins involved in carbohydrate metabolism during the adaptation period following hyperosmotic shock (i.e. at time 0, 30 and 80 min after the shift). Only three genes encoding the enzymes in the glycolysis/gluconeogenesis pathway exhibited a significant change in transcription level, including *fbaB* (4.60 $\log_2$-fold increase), *tpiA* (1.03 $\log_2$-fold decrease) and *ybhA* (1.15 $\log_2$-fold decrease). On the other hand, when the osmotically-treated cells had resumed growth (i.e. at time 310 min), several genes and proteins of the glycolysis/gluconeogenesis pathway appeared to be up-regulated, although significant changes in their overall expression were not evident in the T-profiler analysis (Fig. 4.5). These included *fbaB*/FbaAB, *pfkB*/PfkAB, *mdh*/Mdh and *maeB*/MaeB with the expression level increased by up to 6.40 $\log_2$-fold. Similarly, a number of genes and proteins involved in the pentose phosphate

pathway were up-regulated at this time point. Among these, transaldolase A (*talA*/TalA) and transketolase B (*tktB*/TktB), which are involved in the non-oxidative branch of the pentose phosphate pathway, exhibited a significant up-regulation. Regulation of these genes is also known to be positively controlled by RpoS at the transcriptional level (see below) (Weber et al. 2005).

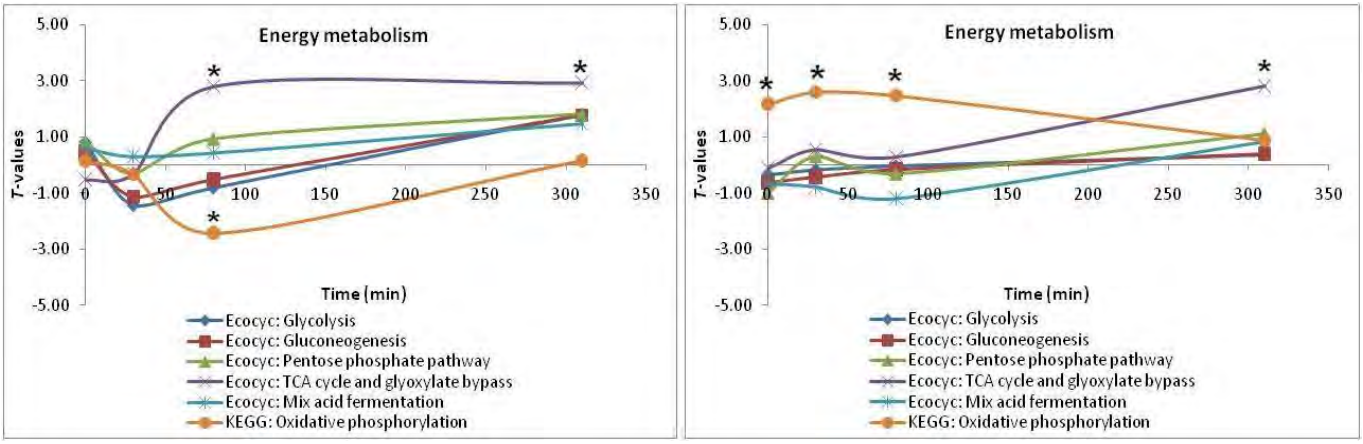

Fig. 4.5. Changes in overall expression of genes (left panel) and proteins (right panel) involved in energy metabolism during exposure of *E. coli* O157:H7 Sakai to hyperosmotic shift from a_w 0.993 to a_w 0.967, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated databases, as described in Section 4.2.11. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

E. coli typically utilizes three alternative modes for energy generation, including aerobic respiration, anaerobic respiration and fermentation (Pin et al. 2009). It has been shown that the aerobic respiration of *E. coli* upon exposure to hyperosmotic shock is severely inhibited as a consequence of plasmolysis, and that the respiratory activity is recovered during deplasmolysis (Meury 1994). In the present study, the transcriptomic and proteomic data were inconsistent with regard to responses related to oxidative phosphorylation (Fig. 4.5). A number of genes encoding

the enzymatic complexes involved in oxidative phosphorylation were down-regulated at 80 min of the treatment, as indicated by a significant negative *T*-value of 2.43. These included genes *nuoKLMN* (1.23 $\log_2$-fold decrease on average) encoding the membrane components of NADH:ubiquinone oxidoreductase I. By contrast, the proteomic analysis indicated a significant increase in overall abundance of proteins involved in oxidative phosphorylation within the first 80 min. A common set of proteins observed at these time points were the components of NADH:ubiquinone oxidoreductase I (NuoCFI; 2.94 $\log_2$-fold on average), succinate dehydrogenase (SdhA; 2.77 $\log_2$-fold on average), and ATP synthase (AtpB; 3.32 $\log_2$-fold on average). These inconsistent observations from two different methodologies may be in line with those of Gygi et al. (1999) who compared mRNA and corresponding protein levels, as described above. However, *E. coli* appeared to increase aerobic respiration after prolonged exposure to hyperomsotic stress, as indicated by significant positive *T*-values for the TCA cycle and glyoxylate bypass pathways (Fig. 4.5). The transcriptomic study revealed a significant increase in expression of several genes (*aceAB*, *acnA*, *sdhACD* and *gltA*; 3.49 $\log_2$-fold on average) encoding the enzymes in the TCA cycle and glyoxylate bypass at 80 and 310 min of the stress, whereas almost all the TCA cycle and glyoxylate bypass-related proteins were found to be significantly up-regulated only at time 310 min in the proteomic analysis (Appendix F.3).

Taken together, the increased expression of genes and/or proteins involved in the major processes of carbohydrate catabolism and energy generation at 310 min after the downshift may be to compensate for the reduction in activity of these processes at low a_w, or may reflect a high level of energy production together with an increase in level of precursors for the biosynthesis of various macromolecules. The latter corresponds well with the observation that *E. coli* resumed

their growth at or before this time point (Fig. 4.3). A recent study has also shown an increase in metabolic activities to meet an energy requirement during exponential growth at high osmolality (25°C a_w 0.967) (Kocharunchitt et al. 2011).

4.3.2.3 Two-component regulatory system

The mechanisms involved in osmosensing and subsequent regulatory responses in *E. coli* are strongly controlled by two-component regulatory systems (Aertsen and Michiels 2004; Chung et al. 2006). It is known that the EnvZ/OmpR system regulates the transcription of genes encoding porins OmpC and OmpF in response to changes in medium osmolality. This regulation is characterized by an altered ratio of these porins, whereby the synthesis of OmpC increases, and that OmpF decreases under hyperosmotic stress (Pratt et al. 1996; Zhou et al. 2003). However, a strong activation of this system was not evident in this study (Appendix F.3), although a significant increase in the abundance of OmpC and OmpR was observed at time 0 and 310 min of the osmotic stress, respectively.

4.3.2.4 Amino acids biosynthesis

Biosynthetic genes and proteins for various amino acids had altered expression levels after an abrupt downshift in a_w (Appendix F.3 and Fig. 4.6). Among these, the mRNA level of *his*CDF, which encode key enzymes involved in histidine biosynthesis, was significantly reduced at 80 min of the shift (up to 1.14 $\log_2$-fold). This was accompanied by the observation that the T-profiler analysis revealed a significant decrease in the overall expression of histidine biosynthetic genes at this time point (Fig. 4.6). The biosynthesis of histidine is known to be an energetically expensive process, requiring 41 ATP molecules per histidine molecule made (Brenner and Ames

1971). Previous studies have also demonstrated that constitutive expression of the *his* operon caused a number of physiological changes in *E. coli*, including growth inhibition in high-salt media (Casadesus and Roth 1989; Frandsen and D'Ari 1993). Therefore, the apparent decrease in the activity of histidine biosynthetic pathway might indicate that *E. coli* suppresses this biosynthetic pathway to prevent an inappropriate use of the cellular energy, possibly for re-allocating the energy to repair cell damage as observed during the recovery period (i.e. at time 80 min) (Fig. 4.3).

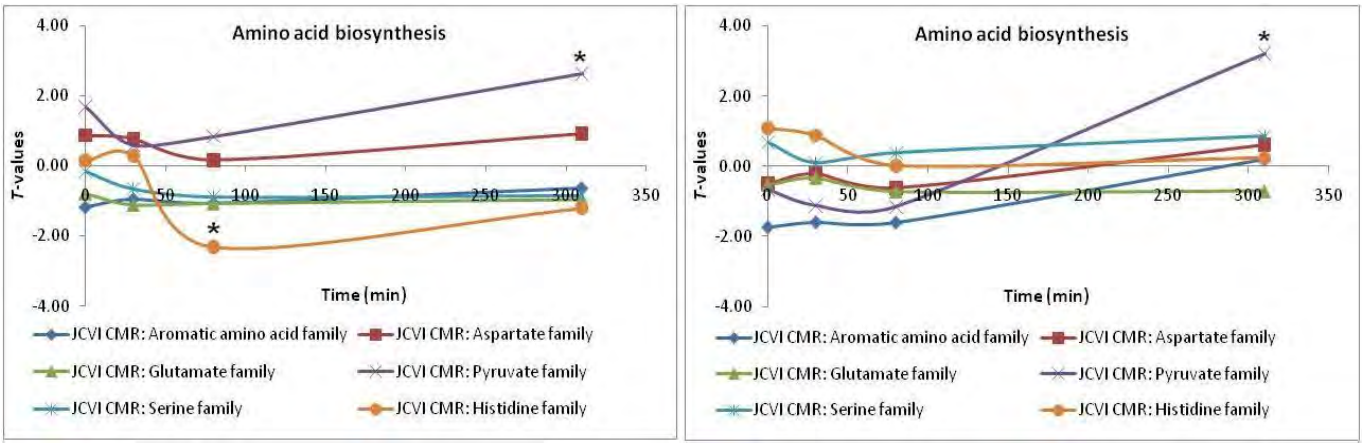

Fig. 4.6. Changes in overall expression of genes (left panel) and proteins (right panel) involved in amino acid biosynthesis during exposure of *E. coli* O157:H7 Sakai to hyperosmotic shift from a_w 0.993 to a_w 0.967, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated database, as described in Section 4.2.11. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

On the other hand, the present study observed an induced activity of the methionine biosynthetic pathway in osmotically-adapted cells (i.e. at 310 min of the treatment), as indicated by a significant up-regulation of *metEH* genes and MetBEH proteins (an increase in abundance of methionine transporters was also observed, see below). Methionine is required for initiation and

elongation of proteins, biosynthesis of polyamines, purines and pyrimidines, and various methylation reactions (Ron et al. 1990; Gur et al. 2002). Previous studies have also demonstrated that the growth of *E. coli* at elevated temperatures is limited by the availability of endogenous methionine (Ron et al. 1990), and that the supplementation of methionine into the growth medium causes the development of ethanol stress tolerance in yeast *Saccharomyces cerevisiae* (Hu et al. 2005). This has led to the suggestion that an increase in the cellular level of methionine might contribute to the maintenance of balanced growth rate of *E. coli* at high osmolality. However, Kocharunchitt et al. (2011) did not find up-regulation of the methionine biosynthetic pathway during growth at a similar osmotic stress condition of 25°C a_w 0.967. This might be due to the differences in the condition tested in the experiment described here (i.e. 35°C a_w 0.967). Furthermore, the T-profiler analysis revealed a significant increase in overall expression of genes and proteins responsible for the biosynthesis of amino acids in the pyruvate family at the time point at which osmotically-treated cells resumed their growth after osmotic upshift (i.e. at time 310 min) (Fig. 4.6). Among these, branched-chain amino acids biosynthetic pathway genes and proteins (*ilvBDG*/IlvBDG) exhibited a significant up-regulation. This agrees well with the observations on the adaptive response of *E. coli* to osmotic stress (25°C a_w 0.967) (Kocharunchitt et al. 2011). Taken together, the apparent increase in expression of several genes and proteins related to the amino acid biosynthetic pathways (i.e. methionine, isoleucine and valine) supports the previous hypothesis that an increase in intracellular contents of these amino acids may be part of the responses to enhance growth and/or survival of *E. coli* under osmotic stress (Kocharunchitt et al. 2011). Horinouchi et al. (2010) have also shown that supplementing culture media with specific amino acids (i.e. tryptophan, histidine and branched-chain amino acids)

promoted the growth of *E. coli* under ethanol stress, presumably by enabling the culture to develop ethanol tolerance.

4.3.2.5 Transport functions

Accumulation of compatible solutes through uptake from the external environment by active transport is one of the most important responses for bacteria to counter hyperosmotic stress (O'Byrne and Booth 2002). Indeed, the present study found a significant increase in activity of a number of transporters for compatible solutes upon exposure to osmotic upshift (Appendix F.3). Despite that proteins involved in the high-affinity potassium ion (K^+) transport system (Kdp) were not detected in this study, abundance of the low-affinity system Kup, was up-regulated by up to 3.57 $\log_2$-fold at all time points. The potassium channel protein Kch also exhibited an increase in its abundance (2.55 $\log_2$-fold on average) at time 80 min and 310 min. Accordingly, the expression of genes (*gltJI*) encoding transporters for glutamate (the potassium ion counter ion) was found to be induced at these time points (1.47 $\log_2$-fold on average). Other transport systems that were up-regulated in both transcriptomic and proteomic analyses included those responsible for the uptake of glycine betaine/proline (*proVWX* operon; at 30, 80 and 310 min of the treatment) and other osmoprotectants (*yehWXYZ* operon; at 80 and 310 min). In addition, NhaAB sodium ion/proton antiporters exhibited a significant increase in their abundance throughout the course of osmotic stress. These antiporters are known to be implicated not only in osmotic adaptation, but also pH homeostasis (Wood 1999).

Apart from the uptake of several compatible solutes as already mentioned, exposure of *E. coli* to a sudden downshift in a_w also caused a significant alteration in the activity of other groups of

transport system. It was observed that abundance of several transport proteins for phosphate and amino acid was induced. These included the proteins involved in the uptake of arginine (ArtQMP; at 310 min of the treatment) and methionine (MetQN; at all time points). For the group of peptide and nickel transporters, production of SapCF, which are components of the SapABCDF uptake system for peptides were induced at 30 and 310 min of the a_w treatment. Proteins involved in the transport of murein oligopeptides (OppBD) also exhibited a significant increase in abundance at time 310 min.

The transcriptomic analysis revealed a significant down-regulation of several genes, encoding transporters for minerals, and inorganic and organic ions, such as sulfate/thiosulfate (*cysWP*; at 80 min after a_w downshift) and molybdate (*modABC*; at 310 min). Transcription of the *potAB* operon involved in the uptake of polyamines, putrescine and spermidine was also reduced at 80 and 310 min following the shift. This may indicate a decrease in the cellular level of polyamines. Lack of polyamines has previously been shown to be linked to abnormal growth and oxidative stress-induced damage (Jung et al. 2003). Furthermore, genes encoding monosaccharide and oligosaccharide transporters were found to be up-regulated. These included those responsible for the uptake of xylose (*xylFHG*; at 80 min) and maltose (*malEGFK*; at 310 min). It has previously been reported that catabolism of maltose can contribute indirectly to enhance the levels of endogenously synthesized osmolytes such as glutamate and *N*-acetylglutaminylglutamine amide, facilitating growth at elevated osmolarities (Sleator and Hill 2001).

Increased production of several proteins associated with the uptake system for metallic cations, iron-siderophores and vitamin B12 was observed in response to osmotic upshift. TonB and

ExbBD, which make up the TonB-ExbBD energy transducing system to provide the energy source required for the uptake of iron-siderophore complexes and vitamin B12 across the outer membrane, were significantly up-regulated throughout the treatment. This was accompanied by the observations that FhuCD were significantly up-regulated (4.42 $\log_2$-fold on average) at all time points, indicating increased activity of the TonB-dependent iron (III) hydroxamate transport system. Abundance of FepA, a subunit of the TonB-dependent uptake system for ferric enterobactin also appeared to be significantly induced (2.63 $\log_2$-fold on average) within the first 30 min of the shift. In keeping with these findings, the response of *E. coli* during steady-state growth at a similar stress condition (25°C a_w 0.967) has previously revealed an increase in overall abundance of proteins involved in the transport of cations and iron compounds (Kocharunchitt et al. 2011). The enhancement of iron uptake observed here may be associated with an increase in levels of intracellular hydrogen peroxide (H_2O_2) and superoxide (O_2^-), which invoke the oxidative stress response (Horinouchi et al. 2010). This also corresponds well with the present observations in which a number of genes and proteins involved in defense mechanism against oxidative stress were significantly up-regulated by osmotic stress (see below). In addition, BtuE a component of the TonB-dependent vitamin B12 transport system exhibited a significant increase in its abundance of up to 2.45 $\log_2$-fold within the first 80 min of the stress.

4.3.2.6 Fatty acids and lipids metabolism

E. coli membranes typically comprise three major phospholipids, including phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL). Growth at low a_w typically leads to changes in membrane lipid composition by increasing the proportion of anionic (PG and CL) over zwitterionic (PE) phospholipids. Such changes are to prevent the

membrane lipids from adopting a non-bilayer phase, which otherwise could disrupt membrane function (Russel et al. 1995; Romantsov et al. 2008). However, both transcriptomic and proteomic data did not provide strong evidence supporting this adaptive strategy. A significant increase in overall abundance of enzymes in the biosynthetic pathway of phospholipids was observed within the first 30 min of imposition of the osmotic stress (Fig. 4.7). Among these enzymes, only phosphatidylserine synthase (PssA) and cardiolipin synthase (Cls) were found to be significantly up-regulated (2.94 $\log_2$-fold on average). PssA and Cls proteins are responsible for the *de novo* synthesis of PE and CL, respectively, suggesting that increased production of these phospholipids occurs. Despite that the physiological role of PssA under osmotic stress is unclear, PE has previously been shown to be important for acid adaptation (Canet et al. 2003).

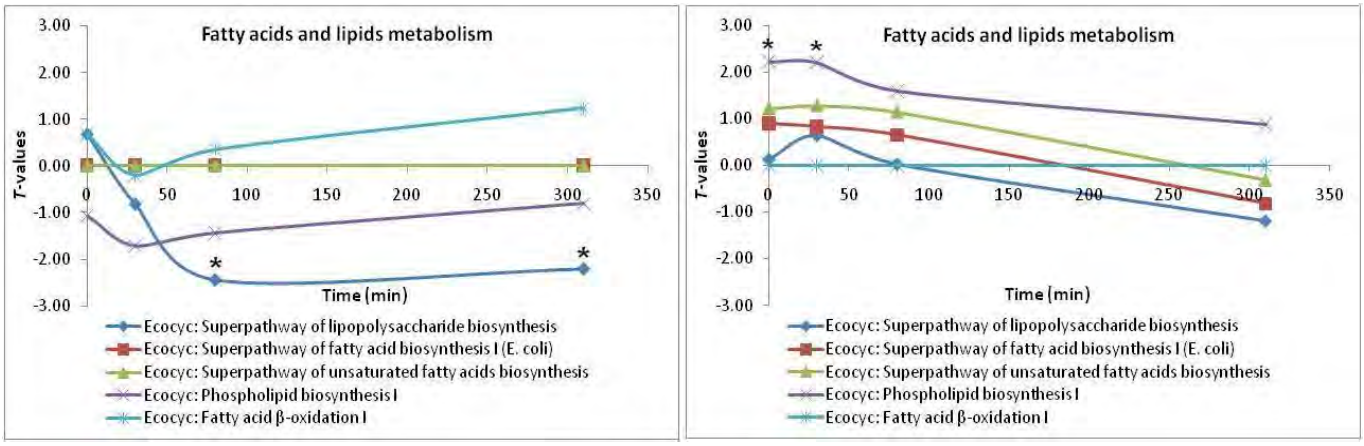

Fig. 4.7. Changes in overall expression of genes (left panel) and proteins (right panel) involved in fatty acids and lipids metabolism during exposure of *E. coli* O157:H7 Sakai to hyperosmotic shift from a_w 0.993 to a_w 0.967, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated database, as described in Section 4.2.11. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

Hyperosmotic stress was found to have no significant effects on overall expression of genes and proteins involved in the major pathways for fatty acid biosynthesis (Fig. 4.7). This was despite the observation that the abundance of FabZ was significantly induced by up to 4.03 $\log_2$-fold at all time points of the stress. The FabZ enzyme plays an important role in the biosynthetic pathways of both saturated and unsaturated fatty acids. In contrast, the transcriptomic analysis revealed a significant up-regulation of the *cfa* gene, which encodes cyclopropane fatty acyl phospholipid synthase at time 80 and 310 min. The increase in cyclopropane fatty acid content in the cell membrane has previously been demonstrated to assist cells to maintain intracellular pH homeostasis by reducing membrane permeability to protons. This lipid modification probably provides great protection against acid stress and other stress conditions such as high salt concentration and ethanol (Brown et al. 1997; Chang and Cronan 1999; Shabala and Ross 2008). Furthermore, the observations on fatty acid composition are consistent with the previous study on the response of *Lactococcus lactis* to osmotic stress, in which this organism increases the level of cyclopropane fatty acids, whereas the unsaturated-to-saturated fatty acids ratio remains unchanged (Guillot et al. 2000).

Several genes, encoding the key enzymes for lipopolysaccharide biosynthesis (i.e. *lpxH* and *waaA*) were down-regulated with a significant negative *T*-value at 80 and 310 min after hyperosmotic shift (Fig. 4.7). The apparent reduction of lipopolysaccharide biosynthesis might indicate that outer membrane instability occurs during adaptation to hyperosmotic stress. Consistent with this, previous studies have reported that a defect in lipopolysaccharide biosynthesis leads to the lack of a continuous lipopolysaccharide layer in the outer membrane, causing increased susceptibility of bacterial cells to hydrophobic antibodies (Vuorio and Vaara

1992; Vaara and Nurminen 1999). Furthermore, it has been demonstrated that the envelope stress caused by the defective biosynthesis of lipopolysaccharide increases the biosynthesis of the exopolysaccharide colanic acid (Majdalani et al. 2005; Meredith et al. 2007). This, indeed, agrees well with earlier observations, indicating that the Rcs system-regulated colanic acid biosynthesis becomes activated (see Section 4.3.2.7).

4.3.2.7 Cell structure components

During adaptation to hyperosmotic stress, *E. coli* up-regulated several genes and proteins involved in the Rcs phosphorelay system that regulates the biosynthesis of colanic acid (Hagiwara et al. 2003) (Fig. 4.8). The T-profiler results revealed that a significant increase in overall expression of genes previously known to be induced by Rcs regulon (Hagiwara et al. 2003) occurred from 30 min of the osmotic treatment onward, whereas several Rcs-dependent proteins were significantly up-regulated only at the time point at which *E. coli* had resumed growth (i.e. at time 310 min). These genes and proteins were also found to be amongst the most highly up-regulated in the present study (Appendix F.3). Kocharunchitt et al. (2011) demonstrated strong up-regulation of Rcs-dependent elements together with a high level of colanic acid production in *E. coli* cells grown under a similar stress condition (25°C a_w 0.967). The importance of colanic acid has frequently been described in protecting cells against a variety of stresses, including osmotic stress (Ophir and Gutnick 1994; Chen et al. 2004; Mao et al. 2006), and has been shown to be involved in biofilm formation (Danese et al. 2000). Although the physiological role of colanic acid is not well understood, it is thought that colanic acid expressed on cell surfaces simply provides a physical barrier to protect cells from hostile environments (Sledjeski and Gottesman 1996; Mao et al. 2006). Allen et al. (1987) has reported

that colanic acid confers a strong negative charge to the cell surface. This negativly charged cell surface has led to the suggestion that colanic acid may help *E. coli* to maintain hydration of the cell surface, and to preserve the membrane lipids in the proper bilayer phase, as part of the adaptive strategies in response to such stress (Russel et al. 1995; Sleator and Hill 2001). However, the findings of Kocharunchitt et al. (2011) indicated that colanic acid is not required for growth and survival under osmotic stress. A comprehensive understanding on the function of colanic acid is, therefore, needed to explain the induced activity of the Rcs system-controlled colanic acid biosynthesis under osmotic stress condition.

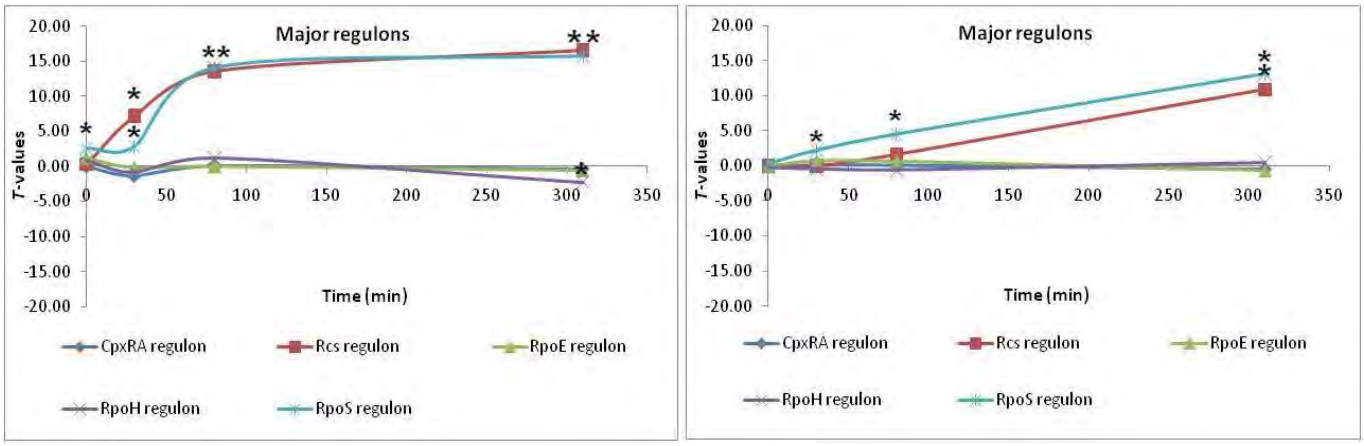

Fig. 4.8. Changes in overall expression of genes (left panel) and proteins (right panel) involved in the major regulons during exposure of *E. coli* O157:H7 Sakai to hyperosmotic shift from a_w 0.993 to a_w 0.967, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the list of genes or proteins previously known to be induced by major regulons, as described in Section 4.2.11. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

4.3.2.8 Bacterial chemotaxis and motility

An immediate increase in overall expression of genes and proteins involved in chemotaxis and motility was observed after osmotic upshift, as indicated by significant positive *T*-values (Fig.

4.9). This was followed by a general decrease in their level over the course of osmotic treatment. Accordingly, the transcriptomic data revealed a significant down-regulation of *rpoF*, which is a sigma factor responsible for controlling the transcription of genes involved in chemotaxis and motility (Chung et al. 2006), at 80 and 310 min of the treatment. It was also found that chemotaxis and motility genes and proteins were amongst the most heavily down-regulated in osmotically-adapted cells (i.e. at time 310 min) (Appendix F.3). Kim et al. (2005) has also reported a transient increase in expression of flagella biosynthetic elements in response to osmotic shock. This induced activity may be an initial response for cells to move away from osmotic stress (Wecker et al. 2009). Furthermore, the strong down-regulation of chemotaxis and motility systems observed here supports recent suggestions in which these systems are the most dispensable functions in *E. coli* during steady-state growth at high osmolality (25°C a_w 0.967) (Kocharunchitt et al. 2011). This adaptive response of *E. coli* is in keeping with the fact that expression of genes encoding flagella are negatively controlled by the master regulator RpoS (Patten et al. 2004) and the Rcs system-activated colanic acid biosynthesis (Francez-Charlot et al. 2003).

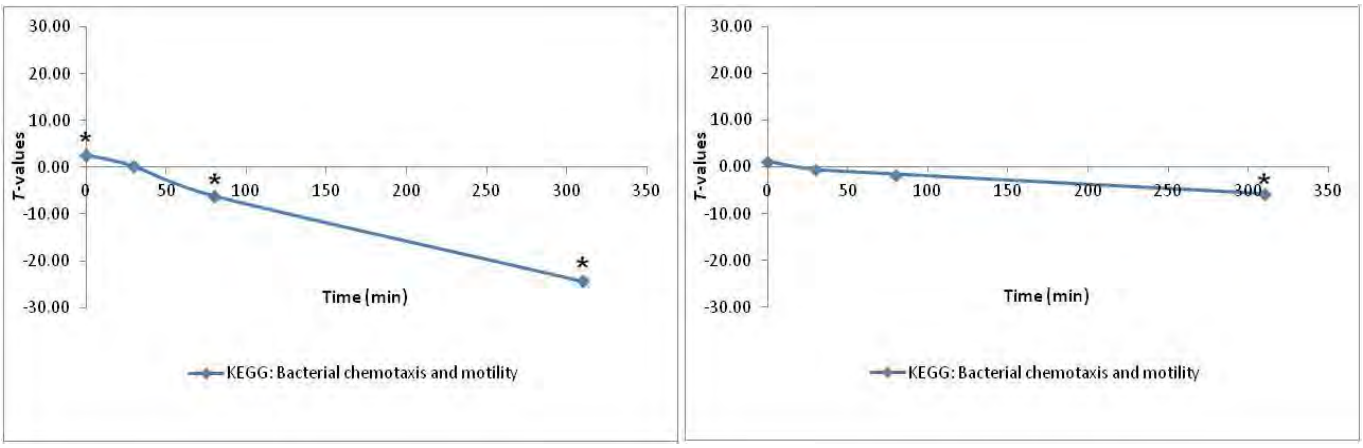

Fig. 4.9. Changes in overall expression of genes (left panel) and proteins (right panel) involved in bacterial chemotaxis and motility during exposure of *E. coli* O157:H7 Sakai to hyperosmotic shift from a_w 0.993 to a_w 0.967, as determined by T-profiler analysis. The predefined set used for T-profiler analysis was based on the indicated database, as described in Section 4.2.11. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

4.3.2.9 Stress response

Exposure of *E. coli* to a sudden downshift in a_w resulted in induced activity of the master stress regulator RpoS. This was evident in the transcriptomic data in which the mRNA level of *rpoS* significantly increased up to 3.40 $\log_2$-fold from 30 min of the shift onward, whereas the proteomic analysis revealed a significant up-regulation of RpoS only at time 80 and 310 min (4.66 $\log_2$-fold on average). Accordingly, the T-profiler analysis of both data showed a large increase in expression of several genes and proteins previously known to be induced by the RpoS regulon (Lacour and Landini 2004; Patten et al. 2004; Weber et al. 2005) over the course of treatment (Fig. 4.8). Many of these have been described to be involved in osmoprotection (Weber and Jung 2002; Weber et al. 2006), including osmotically inducible proteins (*osm* operon; at 30, 80 and 310 min of the treatment); *dps*/Dps, which is responsible for the protection of DNA (at 80 and 310 min); *otsAB*/OtsAB, which are involved in the *de novo* synthesis of

trehalose (at 80 and 310 min); and a group of elements with undefined functions (*elaB*/ElaB, *yciEF*/YciEF, *ygaM*/YgaM and *yjbJ*/YjbJ; at 80 and 310 min). The transcriptomic study also found that a number of RpoS-dependent genes with increased expression at 80 and 310 min of the treatment are known to be associated with defense mechanisms against other stresses (Zheng et al. 2001; Weber et al. 2005). These included genes within the acid resistance fitness island (*gadABWX* and *hdeBD*), and those involved in response to oxidative damage (*katE* and *sufABD*). The proteomic analysis, however, revealed up-regulation of proteins involved in acid (GadAB; 2.47 $\log_2$-fold on average) and oxidative stress responses (KatE and SufAD; 5.35 $\log_2$-fold on average) only at time 310 min. Taken together, these observations support previous reports, demonstrating that the general response network established by the RpoS regulon typically provides cross-protection against diverse stress conditions (Lacour and Landini 2004; Patten et al. 2004; Weber et al. 2005; Kocharunchitt et al. 2011).

Apart from activation of the RpoS regulon as described above, *E. coli* cells transiently induced additional stress responses or those elements controlled by other regulons (i.e. the RpoE and RpoH regulatory systems) during adaptation to hyperosmotic stress (Appendix F.3). A significant up-regulation of *rpoE*/RpoE was observed at 30 min (3.19 $\log_2$-fold; in the proteomic analysis) and 80 min (2.45 $\log_2$-fold; in the transcriptomic analysis) of the osmotic stress, while the mRNA level of *rpoH* significantly increased (1.81 $\log_2$-fold on average) at time 30 and 80 min. The RpoE and RpoH regulons are respectively known to be responsible for the control of protein misfolding in the cell envelope and cytoplasm, and it has been reported that the transcription of the *rpoH* mRNA is RpoE-dependent (Alba and Gross 2004; Nonaka et al. 2006). Furthermore, the majority of genes whose expression is under the control of RpoH were found to

be significantly up-regulated during the recovery period upon hyperosmotic shock (i.e. at time 80 min). These included those encoding molecular chaperones (*dnaK, htpG* and *ibpAB*; 2.51 $\log_2$-fold on average), which have function in facilitating protein re-folding and stabilization, and preventing aggregation of misfolded proteins. DnaK is a member of the major DnaK/DnaJ/GrpE chaperone system, and has previously been reported to be involved in adaptation to osmotic and oxidative stresses (Meury and Kohiyama 1991; Pin et al. 2009). Although other chaperone proteins (i.e. HtpG and IbpAB) have only minor activity in *E. coli* (Thomas and Baneyx 2000), previous studies have described that they play a significant role in *de novo* protein folding under stressful conditions, presumably by expanding the activity of the major system to interact with newly synthesized polypeptides (Bianchi and Baneyx 1999; Thomas and Baneyx 2000).

The apparent response of *E. coli* cells to osmotic upshift through a transient induction of the RpoE and RpoH systems, and activation of the RpoS regulon reflects their robust ability to activate multiple defense mechanisms, and may also underly the observation of a complex pattern of microbial growth behavior in response to such stress. In support of this, it has previously been suggested that increased activity of the RpoE and RpoH regulons may serve as a multipurpose emergency response to repair protein misfolding and facilitate the proper folding of newly synthesized proteins after loss of turgor (as would be expected during the injury and recovery period), while simultaneously activating the master stress regulator RpoS to mediates the long-term adaptation at high osmolality (Bianchi and Baneyx 1999; Kocharunchitt et al. 2011).

4.4 CONCLUDING REMARKS

The present study provided a comprehensive insight into the growth kinetics and molecular response of exponentially growing *E. coli* O157:H7 Sakai subjected to a sudden downshift in water activity. Exposure of *E. coli* to the abrupt osmotic upshifts caused a growth interruption with a complex pattern of microbial population behavior. Immediately after a shift to low a_w, there was a decrease in bacterial numbers followed by a period in which culturable cells rapidly recovered, and thereafter, exponential population growth occurred at a characteristic rate under the given stress condition. The growth characteristics obtained, such as generation time, lag time and adaptation time were found to depend on the magnitude of the shift. However, estimates of RLT, i.e. dividing LT or AT by GT give rise to different responses. The $RLT_{LT/GT}$ response suggests that more adaptation *work* is required as a_w decreases, whereas $RLTs_{AT/GT}$ implied a similar amount of *work* needed for *E. coli* to adapt to a given decrease in a_w.

In an integrated genomic and proteomic study, *E. coli* was found to respond to a rapid downshift at 35°C from a_w 0.993 to a_w 0.967 by evoking a highly complex regulatory process involving expression changes of different groups of genes and proteins. A number of these genes and proteins are known to be involved in well-established adaptive responses to hyperosmotic stress, including those elements responsible for accumulation of compatible solutes. Apart from this, *E. coli* also altered expression of other elements, which are associated with several functional groups and metabolic pathways, to contribute to different stages of osmotic adaptation. It was found that phospholipids biosynthesis exhibited up-regulation during the initial decline in cell numbers after hyperosmotic shift. This implies that cells may modify their membrane lipid composition to preserve membrane functions (possibly for maintaining the transport of

compatible solutes). From the recovery period onward, both Rcs system-regulated colanic acid biosynthesis and enzymes involved in fatty acid biosynthesis appeared to be induced, suggesting their possible role in strengthening the cell envelope structure. This probably allows cells to recover from injury and survive during exposure to osmotic stress. Finally, the apparent increase in expression of several genes and proteins involved in amino acid biosynthetic pathways (i.e. methionine, isoleucine and valine) and the major processes of carbohydrate catabolism and energy generation (i.e. the glycolysis/gluconeogenesis pathway, the pentose phosphate pathway and the TCA cycle and glyoxylate bypass pathways) during growth at low a_w may be part of the responses to enhance growth and/or survival under such stress. Down-regulation of elements associated with protein synthesis was also observed at the time point at which growth was resumed, corresponding with the observed decrease in growth rate of *E. coli* under osmotic stress. Furthermore, *E. coli* was found to have the ability to activate multiple stress responses by transiently inducing the RpoE and RpoH regulons to repair protein misfolding and facilitate the proper folding of newly synthesized proteins following loss of turgor (as occurred during the injury and recovery period), while simultaneously activating the master stress regulator RpoS to mediate the long-term adaptation at hyperosmolality. This clearly indicates its remarkable adaptability to survive under stress conditions. The results described in this chapter highlight the potential mechanisms enabling survival and growth of *E. coli* during exposure to osmotic stress conditions, and will aid understanding of its physiological response to simultaneous abrupt downshifts in temperature and water activity stress (Chapter 5).

CHAPTER 5

PROTEOMIC RESPONSE OF *ESCHERICHIA COLI* O157:H7 SAKAI DURING DYNAMIC CHANGES IN GROWTH KINETICS INDUCED BY SIMULTANEOUS ABRUPT DOWNSHIFTS IN TEMPERATURE AND WATER ACTIVITY

5.1 INTRODUCTION

Conventional air-chilling of carcasses is used in the meat industry in Australia and New Zealand. It typically involves the exposure of carcasses to a flow of cold air. This leads to a decrease in both temperature and water activity (a_w) at the surface of meat carcass, to levels that have a significant effect on the growth of contaminating bacteria, including *E. coli*. However, these bacteria can sense environmental changes, and induce an adaptive response though a highly complex regulatory process involving expression changes of functionally different groups of genes and proteins. This response allows bacteria to protect their vital processes and restore cellular homeostasis (Aertsen and Michiels 2004; Chung et al. 2006). Shifting bacteria to temperatures below their optimum, particularly below the so-called 'normal range' (Ingraham and Marr 1996) causes a number of physiological and morphological changes. These include alterations in cell membranes by increasing unsaturated fatty acid content, changes in the transcriptional and translational machinery and the production of cold shock and cold acclimation proteins (Yamanaka 1999; Cao-Hoang et al. 2008). In response to hyperosmotic stress (low a_w), bacteria typically activate osmoregulatory systems to restore intracellular osmotic balance to within tolerable limits. This is achieved by active uptake of charged solutes (e.g. potassium ions and glutamate), followed by accumulation of compatible solutes either through *de novo* biosynthesis (e.g. trehalose) or through uptake from the external environment

(e.g. glycine betaine and proline) (O'Byrne and Booth 2002; Chung et al. 2006). Numerous studies have also reported that bacterial cells previously challenged by one environmental stressor acquire increased resistance to other stresses (cross protection). Therefore, the ability of bacteria to adapt to and survive under adverse conditions could increase their potential risk to public health. A comprehensive understanding of the mechanisms of adaptation used by bacteria in response to combined cold and osmotic stress is necessary in aiding the development of more targeted approaches for the meat industry to eliminate or control *E. coli* during carcass processing.

Kocharunchitt et al. (2011) reported an integrated genomic and proteomic approach to investigate the physiological adaptation of *E. coli* O157:H7 strain Sakai during exponential growth under steady-state conditions, relevant to low temperature and water activity conditions experienced during carcass chilling. Growth of *E. coli* under combined cold and osmotic stress (14°C a_w 0.967) resulted in activation of the master stress response regulator RpoS and the Rcs phosphorelay system involved in the biosynthesis of the exopolysaccharide colanic acid, as well as down-regulation of elements involved in chemotaxis and motility (Kocharunchitt et al. 2011). Using the same approach, time-dependent alterations in genome and proteome profiles of *E. coli* O157:H7 Sakai in response to a rapid downshift in temperature (from 35°C to 14°C) (Chapter 3) or water activity (from a_w 0.993 to a_w 0.967) (Chapter 4) were studied. It was demonstrated that *E. coli* universally responded to cold shock and hyperosmotic shock by transiently inducing the RpoE regulon, which controls protein misfolding in the cell envelope, while simultaneously activating the master stress regulator RpoS to mediate the long-term adaptation under such stress (Chapters 3 and 4). Those findings provide comprehensive knowledge of the potential

mechanisms enabling survival of this pathogen during exposure to these stress conditions. To gain a complete insight into the physiological response of exponentially growing *E. coli* O157:H7 Sakai to dynamic conditions of temperature and water activity downshift as occur during carcass chilling, the work described in this chapter was undertaken to investigate the growth kinetics and time-dependent changes in the proteome of exponential phase *E. coli* O157:H7 Sakai subjected to simultaneous sudden downshifts in temperature and a_w from 35°C a_w 0.993 to 14°C a_w 0.967. The proteomic response was analyzed by 2D-LC/MS/MS analysis. Differences in protein abundance in *E. coli* before and after combined cold and osmotic shifts were analyzed through quantitative and comparative analysis of time-dependent changes in protein abundance.

5.2 MATERIALS AND METHODS

5.2.1 Experimental work

All experiments described below were undertaken in the Food Safety Centre, University of Tasmania (Hobart, Australia).

5.2.2 Bacterial strain

E. coli O157:H7 Sakai was used throughout this study (Section 2.2.2).

5.2.3 Preparation of low a_w broth

A brain-heart infusion (BHI) broth (CM225, Oxoid, Australia; Appendix A.7) containing a very high concentration of NaCl (a_w 0.760 ± 0.003) was prepared as described previously (Section 4.2.3). The a_w of this NaCl-concentrated BHI broth was confirmed with an Aqualab CX-2 dew

point instrument (Decagon Devices, Inc., Pullman, USA), before it was used in combined cold and osmotic treatment.

5.2.4 Simultaneous abrupt downshifts in temperature and water activity

A stationary phase culture of *E. coli* (Section 2.2.3) was diluted 1:10^4 in a 25 ml of pre-warmed (35°C) BHI broth. This 'primary' culture was incubated at 35°C with agitation (80 oscillations.min^{-1}) in a water bath (Ratek Instruments, Australia), and its growth monitored turbidimetrically at 600 nm with a Spectronic 20 spectrophotometer (Bausch and Lomb, USA). After achieving an optical density at 600 nm (OD_{600}) of 0.1 ± 0.01 (i.e. the mid-exponential phase of growth, ~10^7 CFU.ml^{-1}), the primary culture was diluted 1:10^2 in a fresh 25 ml of pre-warmed (35°C) BHI broth to prepare a 'secondary' culture. This culture was then incubated at 35°C with agitation until it reached the mid-exponential growth phase (OD_{600} of 0.1 ± 0.01). At this point, the culture was subjected to simultaneous sudden downshifts in temperature and water activity. This was done by adding an appropriate volume of NaCl-concentrated BHI to yield a final a_w of 0.967 and immediately placing the culture in a 14°C water bath with shaking. The water bath was maintained within ± 0.1ºC of the test temperature, and temperature was continuously recorded using type T thermocouples connected to a Grant Squirrel, model 1255, data logger (Grant Instruments, Cambridge, UK).

5.2.5 Microbiological analysis

Growth of *E. coli* was determined periodically by viable count, as described in Section 4.2.5. The growth experiment was performed in quadruplicate at two different times.

Construction of growth curves, as well as analysis of their kinetic parameters (i.e. generation time, GT; lag time, LT; and adaptation time, AT) were carried out, as described in Section 4.2.5. Relative lag time (RLT) was calculated by dividing LT or AT with GT, to estimate the relative amount of *work* to be done by *E. coli* to adjust to new conditions (Robinson et al. 1998; Mellefont and Ross 2003).

Where appropriate, statistical analysis was applied to assess differences in kinetic parameters using Student's *t* test, at a confidence interval of 95% (*P*-value = 0.05).

5.2.6 Proteomic analysis

In an independent set of experiments, abrupt downshifts of nine cultures of *E. coli* from 35°C a_w 0.993 to 14°C a_w 0.967 were performed and processed as described above (Section 5.2.4) to prepare samples for 2D-LC/MS/MS analysis. Each lot of the cell cultures (25 ml) was, respectively, harvested before the downshifts in both temperature and a_w (i.e. control), and at 0 (i.e. immediately after the shifts), 60, 250, 1605, 4070, 5700, 9900 and 18565 min after the shifts. Each sample taken was then subjected to the extraction protocol for soluble and membrane proteins according to Section 3.2.5. Table 5.1 describes the number of biological replicates performed for each time point in the proteomic analysis.

Table 5.1 Number of biological replicates performed in 2D-LC/MS/MS analysis.

Time points	Number of biological replicates[a]
Before combined cold and osmotic shifts	6(6)
Immediately after shifts	2(2)
60 min after shifts	2(2)
250 min after shifts	2(2)
1605 min after shifts	2(2)
4070 min after shifts	2(2)
5700 min after shifts	2(2)
9900 min after shifts	2(2)
18565 min after shifts	2(2)

[a] Number of replicates performed for soluble (outside brackets) and membrane (within brackets) fractions of *E. coli*.

Analysis of 2D-LC/MS/MS was carried out on protein samples as previously described in Section 2.2.12.

5.2.7 MS/MS data analysis

MS/MS spectra were processed and searched against the complete database of *E. coli* O157:H7 Sakai (5,318 entries in total) as described in Section 2.2.13. All protein identifications that passed the criteria (i.e. at PeptideProphet and ProteinProphet of ≥0.9) were then assigned for their confidence level according to Section 3.2.7. Only protein identifications with a 'high' and 'intermediate' confidence level (referred as having a 'high confidence score') were accepted for further analysis.

Following data filtering, all protein identifications with high confidence from membrane and soluble fractions of the same sample were combined to represent a 'total' fraction of the corresponding sample. This approach was performed to improve reproducibility of the proteomic data as described previously (Section 3.2.7). The observed R^2 values, as determined by linear regression analysis, indicated a relatively stronger linear correlation between spectral counts (SpCs, the number of MS/MS spectra) for all possible pair-wise comparison of replicates of

pooled fraction (average $R^2 = 0.92 \pm 0.06$) when compared to that of replicates of each fraction (average $R^2 = 0.74 \pm 0.13$, and 0.88 ± 0.13 for membrane and soluble fractions, respectively). Each of the total fractions across the biological replicates was then used to generate the final list of proteins identified at each sampling time.

5.2.8 Protein abundance ratio and its significance

The spectral count generated by 2D-LC/MS/MS analysis was used as a semi-quantitative measure of protein abundance (Nesvizhskii et al. 2003; Liu et al. 2004). The normalized spectral abundance factor (NSAF) for each protein was then calculated as detailed in Section 3.2.8.

Fold changes in protein abundance due to cold and osmotic shocks were calculated as described in Section 3.2.8. Significance analysis was carried out using the beta-binomial test implemented in R (Pham et al. 2010). All proteins with a *P*-value <0.01 and at least 2-fold change were considered to be differentially expressed.

5.2.9 Proteomic data mining

Information on identified proteins including protein and gene names, ECs numbers (locus tag), GI numbers, NCBI Reference Sequence (RefSeq), protein sizes and molecular masses, as well as their preliminary functions and properties were obtained from public databases as described previously (Section 3.2.9). In the present study, protein name in conjunction with ECs number was used as a unique identifier for identified proteins.

5.2.10 Predefined set enrichment analysis

The T-profiler analysis was performed as described previously (Section 2.2.16) on the $\log_2$ ratios to determine changes in overall abundance of a predefined set of proteins due to combined cold and osmotic shocks. The predefined sets of proteins used for the present analysis were based on the selected functional role categories and/or metabolic pathways of the databases as given in Section 3.2.10. The *T*-value obtained from the analysis was determined only for sets that contained at least five members, and its significance was established by using the associated two-tailed *P*-value. All predefined sets with a *P*-value less than 0.1 were considered to be statistically significant.

5.2.11 Clustering analysis of time-course proteomic data

To determine similarities in protein abundance profiles of *E. coli* during exposure to simultaneous downshifts in temperature and a_w, hierarchical clustering analysis (HCA) was performed on the *T*-values calculated for JCVI CMR functional categories. The T-profiler results for the *E. coli* proteome during steady-state growth at 14°C a_w 0.967 (data of Kocharunchitt et al. 2011) were also included in the clustering analysis for comparison. The degree of similarity was calculated with the Euclidean distance metric and complete linkage as a clustering method, using Cluster 3.0 software (de Hoon et al. 2004). The clustering result was then visualized in TreeView version 1.1.3 (Saldanha 2004).

Proteins with significant changes in abundance at one or more time points were also analyzed using the K-means clustering algorithm together with the principal-component analysis (PCA) to define a set of distinct and representative profiles of protein abundance, as described by Koide et

al. (2006). Specifically, principal-component analysis (implemented in R) was performed on the profile of differentially expressed proteins to determine the number of clusters to be considered. The K-mean clustering analysis was then carried out to group each protein into the cluster in which its abundance pattern most closely matches other proteins in the cluster. The degree of similarity was calculated with the Euclidean distance metric as a clustering method, using Cluster 3.0 software (de Hoon et al. 2004), and the clustering result was visualized in TreeView version 1.1.3 (Saldanha 2004).

5.3 RESULTS AND DISCUSSION

5.3.1 Growth of *E. coli* O157:H7 Sakai upon combined cold and osmotic shocks

Simultaneous sudden downshifts in temperature and water activity were applied to *E. coli* cultures during exponential growth (OD_{600} of ~0.1 ± 0.01) in a nutrient rich medium (from 35°C a_w 0.993 to 14°C a_w 0.967). Following the shifts, a complex pattern of microbial population behavior was observed in which the growth curve can be divided into three successive phases (Fig. 5.1). The first phase is comprised of an initial decline in cell numbers followed by a period in which numbers increased rapidly, while a second decrease in bacterial populations and subsequent 'exponential-like' growth occurred during the second phase. Finally, the third phase is considered as that period when the populations appeared to be constant at a similar level to the starting numbers until 'true exponential' growth was re-established. Because of this irregularly shaped growth curve, modeling the kinetic parameters such as generation time, lag time, adaptation time and relative lag time are difficult. Accordingly, this led to determination of these parameters for each phase of the growth curve, as summarized in Table 5.2. It also should be noted that the lag time response is characterized based on a traditional definition of lag time (i.e.

as defined by the time taken to increase above starting numbers), while the adaptation times are determined visually for each phase, as the time taken for bacterial cells to resume exponential growth characteristic of the culture conditions after applying the downshifts.

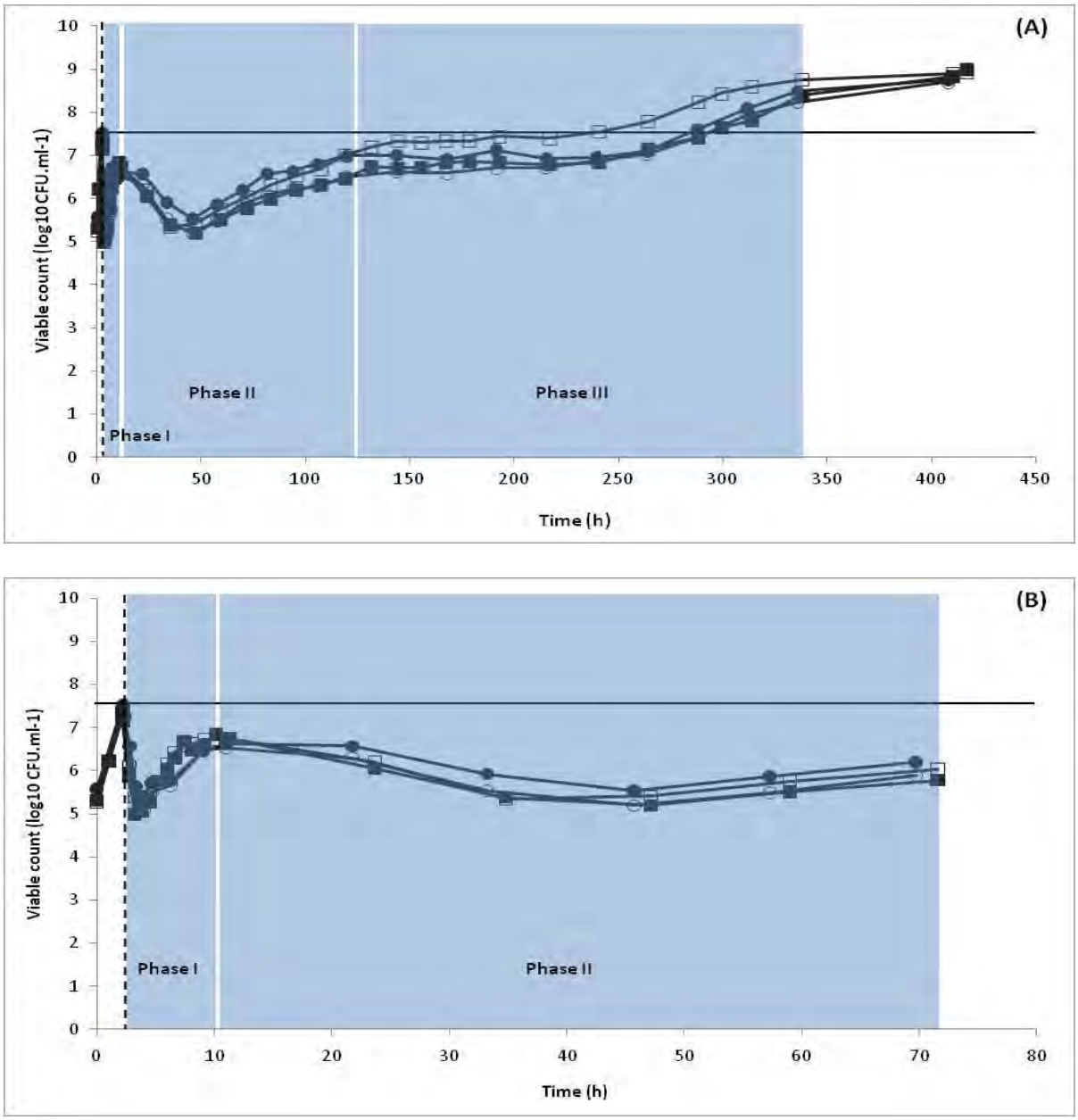

Fig. 5.1. Growth response of *E. coli* O157:H7 Sakai to simultaneous sudden downshifts in temperature and water activity from 35°C a_w 0.993 to 14°C a_w 0.967 for four independent experiments, as determined by viable count. The solid vertical line represents the starting numbers at which simultaneous downshifts in temperature and a_w were applied (a dotted line). Panel (A) shows all available data and panel (B) shows all data on a reduced time scale.

All GT obtained were found to be inconsistent with the GT (8.28 h) predicted by the model of Ross et al. (2003) (Table 5.2). This inconsistency might be due to the fact that the starting inoculum culture used for the experiments was relatively high (i.e. at ~10^7 CFU.ml^{-1}). Such high inoculum size can have potential effects on estimation of GT because only the upper region of the growth curve is measured, which may not be representative of the bacteria at maximum exponential growth (Dalgaard et al. 1994). The estimation could be influenced further by a reduction in the maximum population density under stressful conditions (Krist et al. 1998). Therefore, the use of a lower inoculum would provide a better estimate of GT and, hence, lead to more accurate RLT estimate.

Table 5.2 Summary of the growth responses of exponential phase *E. coli* O157:H7 Sakai to simultaneous sudden downshifts in temperature and water activity from 35°C a_w 0.993 to 14°C a_w 0.967, as determined by viable count.

Trial	Starting number (CFU.ml^{-1})[a]	Phase I			Phase II			Phase III				
		GT (h)[b]	AT (h)[c]	RLT	GT (h)	AT (h)	RLT	GT (h)	AT (h)	LT (h)[d]	RLT	
											AT/GT	LT/GT
1	7.36	0.67	1.10	1.64	17.40	37.00	2.13	17.50	239.00	277.49	13.66	15.86
2	7.30	0.62	1.20	1.94	14.54	35.00	2.41	18.13	229.00	230.34	12.63	12.70
3	7.53	1.44	1.30	0.90	15.36	39.00	2.54	15.52	237.00	282.62	15.27	18.21
4	7.48	1.10	1.40	1.27	16.91	37.00	2.19	17.40	230.00	286.52	13.22	16.47
Mean	7.42	0.96	1.25	1.44	16.05	37.00	2.32	17.14	233.75	269.24	13.69	15.81
SD	0.11	0.39	0.13	0.45	1.33	1.63	0.19	1.13	4.99	26.20	1.13	2.30

[a] The number of *E. coli* at which simultaneous downshifts in temperature and a_w were applied.

[b] Generation time (GT) is determined by linear regression for each phase.

[c] Adaptation time (AT) is estimated visually for each phase, as the time taken for cells to resume the exponential growth after applying the shifts.

[d] Lag time (LT) is calculated by linear regression as the time taken to increase above starting numbers.

Despite the inconsistency between observed and predicted GTs, the present data revealed that the GT estimate for phase I was significantly different (*P*-value <0.05) from those of phases II and

III, whereas there were no significant differences in the GTs between phases II and III (Table 5.2). However, a similar pattern of responses was observed for AT and RLT. Both AT and RLT estimates were significantly different (*P*-value <0.05) between phases. They also appeared to increase when determined at later and later phases in the growth curve. Furthermore, the LT response was found to be significantly longer (*P*-value <0.05) than that of AT for phase III. This observation agrees well with the previous findings in which injured or dead cells caused by stress (as observed in this study, see below) may mask the time when the lag period is actually resolved, causing overestimation of lag time (Chapter 4; Mellefont et al. 2004). However, the estimates of RLT for phase III, as determined by dividing LT or AT with GT did not show statistically significant differences.

To explain the complexity of the growth response of *E. coli* to combined cold and osmotic shocks, it is suggested that the initial decline in cell numbers (i.e. during phase I) might be due to the loss of cell culturability on enumeration media as a result of injury. This loss of culturability was followed by a period of cell recovery at a rate significantly faster than those of phases II and III (Table 5.2). It has also been shown in results presented earlier in this thesis that hyperosmotic shock alone (from a_w 0.993 to a_w 0.967) but not cold shock alone (from 35°C to 14°C) could result in the injury and rapid recovery phenomenon (Chapters 3 and 4). This, therefore, has led to the suggestion that the apparent loss, then recovery, of the culturability observed after combined cold and osmotic shocks may be due to the effects of an abrupt downshift in a_w rather than a downshift in temperature. Interestingly, the insignificant differences in the GTs observed between phases II and III (Table 5.2) indicate that *E. coli* may have already resumed exponential growth from phase II onward. However, the subsequent period in which bacterial numbers

remained constant (i.e. during phase III, see Fig. 5.1), is still unclear at present. These observations have prompted further investigations (possibly using culture-independent methods such as flow cytometry) to fully understand the growth response of *E. coli* to simultaneous cold and osmotic shocks and to confirm the present observations.

5.3.2 Proteomic response of *E. coli* O157:H7 Sakai to simultaneous rapid downshifts in temperature and water activity

To understand the regulatory networks involved in adaptation to both low temperature and osmotic stress, multidimensional LC/MS/MS analysis was undertaken to characterize time-dependent changes in the proteome of O157:H7 Sakai in response to abrupt downshifts in both temperature and a_w from 35°C a_w 0.993 to 14°C a_w 0.967 (Fig. 5.1). Cell samples were harvested before the shifts (i.e. control) and periodically throughout the course of combined cold and osmotic treatment (Fig. 5.2). By using proteomic analysis together with data filtering, protein identifications with high confidence detected in total fractions of *E. coli* at each time point were collated and interpreted (Appendix G.1 and Table 5.3). It was also found that an average of 12.6% ± 4.5% of these proteins across all proteomic datasets was uniquely identified from the membrane fraction. This supports the earlier observations that additional extraction of membrane fractions provided a slight to moderate improvement in proteome coverage (see Sections 2.3.1, 3.3.2 and 4.3.2). In addition, the peptide false-positive discovery rate of each dataset was estimated by dividing the number of spectra matching decoy peptides with the total number of spectra and showed to be less than 5% for all MudPIT runs (Appendix G.2).

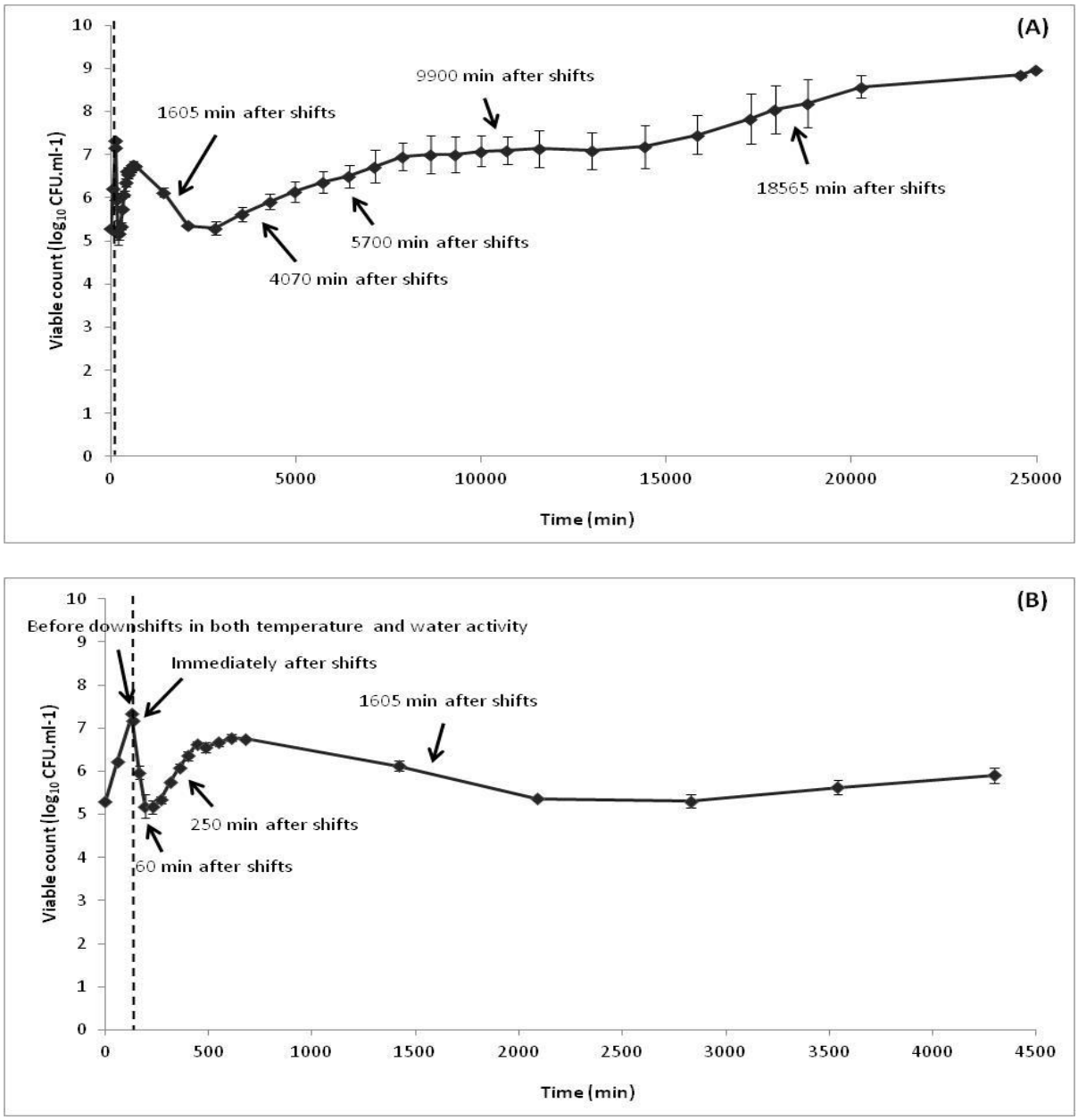

Fig. 5.2. Sampling scheme for analysis of cellular response of *E. coli* O157:H7 Sakai to simultaneous sudden downshifts in temperature and water activity from 35°C a_w 0.993 to 14°C a_w 0.967. The time at which simultaneous downshifts in temperature and a_w were applied is indicated by a dotted line. Block solid arrows indicate the sampling point for 2D-LC/MS/MS analysis. Data points represent means ± standard deviations of at least two independent replicates. Panel (A) shows all available data and panel (B) shows all data on a reduced time scale.

Table 5.3 The number of protein identifications and differentially expressed proteins during exposure of *E. coli* O157:H7 Sakai to combined cold and osmotic stress.

Time points	Number of proteins			Number of differentially expressed proteins[d]
	Membrane fraction[a]	Soluble fraction[a]	total fraction[b]	
Before combined cold and osmotic shifts	343	1,281	1,225(61)[c]	NA[e]
Immediately after shifts	297	553	553(102)	97(55)
60 min after shifts	292	636	631(96)	83(53)
250 min after shifts	168	407	384(56)	44(22)
1605 min after shifts	221	520	490(80)	60(41)
4070 min after shifts	405	688	636(61)	144(71)
5700 min after shifts	455	970	944(65)	334(120)
9900 min after shifts	413	738	733(89)	223(112)
18565 min after shifts	519	777	831(126)	236(132)

[a] Number of proteins identified in membrane and soluble fractions of the *E. coli* proteome that pass the filtering criteria (i.e. at PeptideProphet and ProteinProphet of ≥0.9).

[b] Number of protein identifications with high confidence in total fraction of the *E. coli* proteome (outside brackets).

[c] Number of high-confidence proteins uniquely identified in membrane fraction (within brackets).

[d] Number of proteins with increased (outside brackets) and decreased (within brackets) abundance.

[e] NA; not applicable.

Changes in protein abundance due to combined cold and osmotic stress were determined by comparing the abundance profiles obtained after the shifts to that of a reference sample taken prior to the shifts. Table 5.3 summarizes the number of proteins with significant changes in abundance at each time point. As a result, up-regulation appeared to be dominant over down-regulation at all time points. This observation is consistent with previous study on the molecular response of *E. coli* to a sudden downshift in temperature (Chapter 3) and a_w (Chapter 4). A comprehensive list of differentially expressed proteins with annotations, as well as their comparison to previously published studies is given in the Appendix G.3.

E. coli responded to simultaneous rapid downshifts in temperature and a_w by evoking a highly complex regulatory process involving proteome-wide expression changes of different groups of proteins, as determined by the T-profiler analysis (For comprehensive results see Appendix G.4).

These proteins are known to be associated with a number of functional categories and metabolic pathways, as well as play an important function in the stress responses that are controlled by regulons, as discussed below. Furthermore, clustering analysis of the T-profiler results obtained from this study and that of Kocharunchitt et al. (2011) on the *E. coli* proteome during exponential growth at 14°C a_w 0.967 revealed that protein abundance profiles were generally grouped into two clusters (Fig. 5.3). One cluster was comprised of the protein profiles within the first 1605 min following downshifts in temperature and a_w, while the other consists of those profiles from 4070 min onward and that of steady-state growth at 14°C a_w 0.967. This finding suggests that there were two physiological states of *E. coli* during exposure to combined cold and osmotic shifts. The latter cluster also implies that the physiology of *E. coli* from 4070 min after the shifts onward is similar to that of *E. coli* grown under the condition of 14°C a_w 0.967. This suggests that balanced growth typical of growth at 14°C a_w 0.967 has been established from 4070 min onward, supporting the hypothesis derived from the growth study that *E. coli* might have already resumed growth from phase II of the growth curve onward (see Section 5.3.1). However, the microbial physiology of the subsequent period, in which bacterial numbers were unchanged, is still unclear (i.e. during phase III).

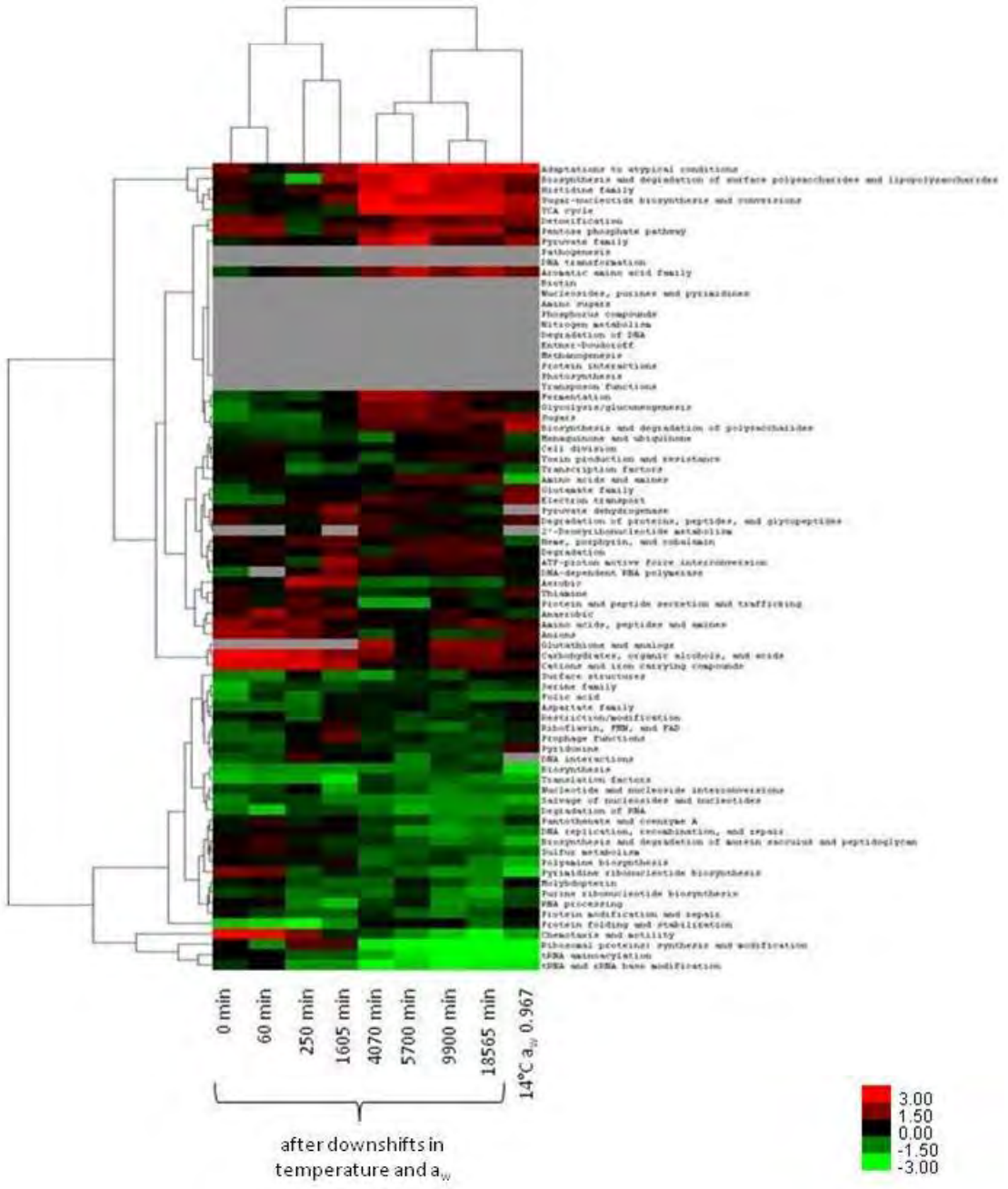

Fig. 5.3. Hierarchical clustering analysis of proteomic data, based on the T-profiler results. The heat map is linked by a dendrogram representing clustering of different protein abundance profiles obtained from *E. coli* during exposure to simultaneous downshifts in temperature and a_w and that during exponential growth at 14°C a_w 0.967 (Kocharunchitt et al. 2011) (top), and *T*-values for JCVI CMR functional categories (side). The color code is as follows: red indicates positive *T*-values; green indicates negative *T*-values; and grey indicates not determined. For a high-quality version of this figure, see Appendix G.5.

5.3.2.1 Transcription and proteins synthesis

Many cellular parameters of bacteria have been identified as functions of the growth rate. The availability of transcriptional and translational machinery in the cell is dependent upon the growth rate in addition to specific regulatory systems (Gyaneshwar et al. 2005; Klumpp et al. 2009). Previous studies have reported that both transcription and translation processes of *E. coli* upon a sudden downshift in temperature and a_w are severely inhibited during the adaptation phase, and these processes are subsequently resumed at a reduced rate when exponential growth occurs under stress conditions (Wood 1999; Phadtare 2004). In the present study, no significant change in overall abundance of RNA polymerases was observed throughout the course of combined cold and osmotic treatment (Fig. 5.4). This was despite that the DNA-directed RNA polymerase subunit omega (RpoZ) was significantly down-regulated within the first 60 min of the treatment, and poly(A) polymerase I (PcnB) exhibited a significant decrease in abundance level from 5700 min onward. By contrast, *E. coli* was found to reduce the amount of translational machinery after prolonged exposure to combined cold and osmotic stress. The T-profiler analysis revealed that several ribosomal proteins were down-regulated with a significant negative *T*-value from 4070 min of the stress onward. This response also corresponds with the period in which *E. coli* had resumed exponential growth after sudden downshifts in temperature and a_w (i.e. from phase II of the growth curve onward; Section 5.3.1). In keeping with this finding, Kocharunchitt et al. (2011) has revealed a reduced capacity of translation in *E. coli* cells during steady-state growth under the same stress condition (14°C a_w 0.967).

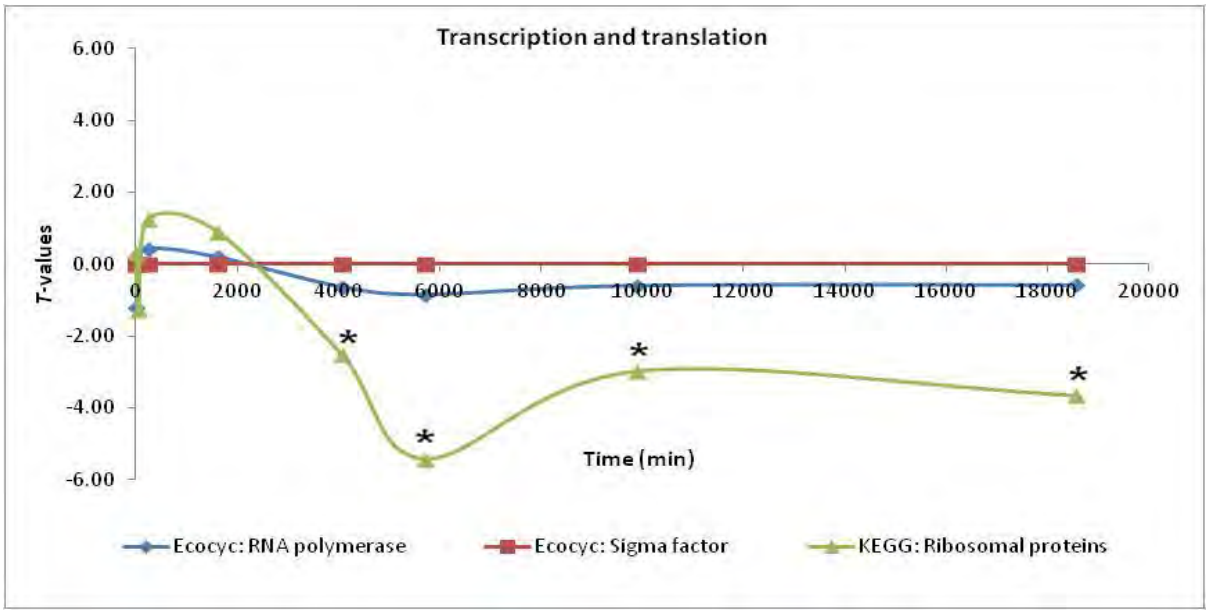

Fig. 5.4. Changes in overall abundance of proteins involved in transcription and translation during exposure of *E. coli* O157:H7 Sakai to combined cold and osmotic shifts from 35°C a_w 0.993 to 14°C a_w 0.967, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated databases, as described in Section 5.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

5.3.2.2 Carbohydrate metabolism and energy generation

Exposure of *E. coli* to simultaneous downshifts in temperature and a_w did not have a significant effect on abundance of proteins involved in the major processes of carbohydrate catabolism, as determined by the T-profiler analysis (Fig. 5.5). This was despite that there are some notable trends observed in abundance level of these proteins (Appendix G.3). The present study found that several enzymes of the glycolysis/gluconeogenesis pathway were significantly down-regulated during the initial decline in cell numbers (i.e. within the first 60 min following the shifts). These include Fbp (at time 0 min), GpmI (at time 0 min), GpmA (at time 0 and 60 min) and FbaA (at time 60 min) with the abundance of up to 5.06 $\log_2$-fold decrease. On the other hand, *E. coli* appeared to up-regulate a number of proteins in the glycolysis/gluconeogenesis

pathway from 4070 min of the combined cold and osmotic treatment onward, including FbaB, Mdh and MaeB (3.59 $\log_2$-fold on average). A similar pattern of response was also observed for the proteins involved in the pentose phosphate pathway, in which both TalA and TktB exhibited a significant increase in abundance level of up to 7.68 $\log_2$-fold from 5700 min of the treatment onward. These proteins are known to be part of the non-oxidative branch of the pentose phosphate pathway, and the observed increase in their abundance is in keeping with the fact that their transcription is positively regulated by RpoS (see below) (Weber et al. 2005).

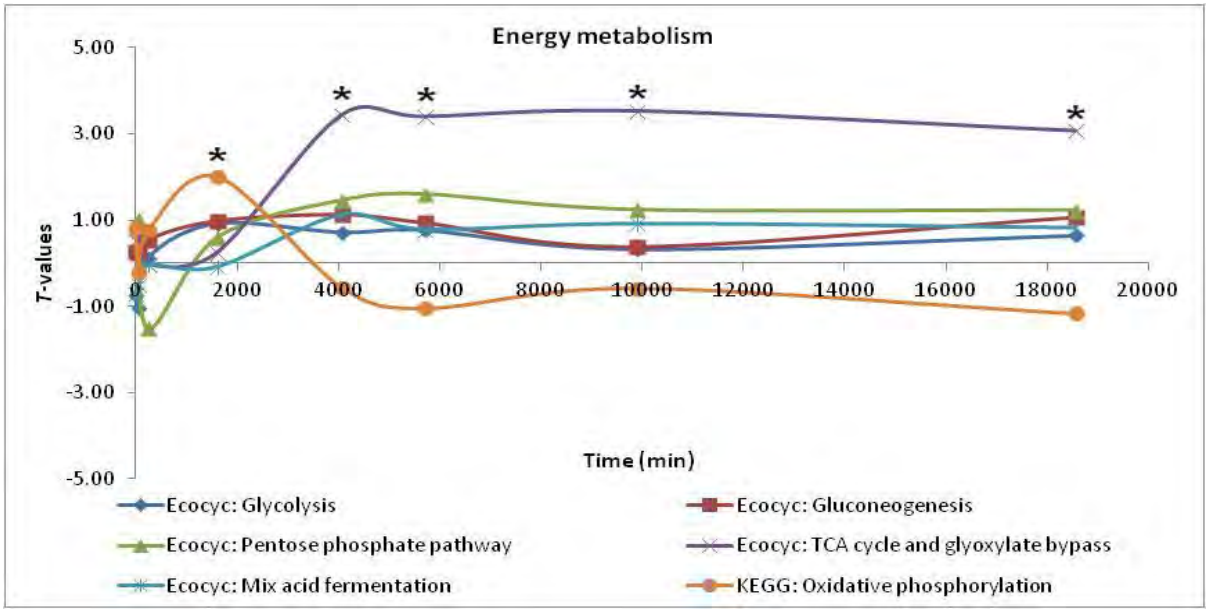

Fig. 5.5. Changes in overall abundance of proteins involved in energy metabolism during exposure of *E. coli* O157:H7 Sakai to combined cold and osmotic shifts from 35°C a_w 0.993 to 14°C a_w 0.967, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated databases, as described in Section 5.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

Previous studies have shown that respiration of *E. coli* occurs at a reduced rate under hyperosmotic stress (Wood 1999), and that oxygen consumption rates appear to be lower at low

temperatures (Gadgil et al. 2005). However, the present data indicate that *E. coli* increased aerobic respiration after prolonged exposure to combined cold and osmotic stress. It was found that almost all the TCA cycle and glyoxylate bypass-related proteins were up-regulated with significant *T*-values from 4070 min of the stress onward (Fig. 5.5). These include AcnA, GltA, IcdA, Mdh, SdhA and SucBCD (3.18 $\log_2$-fold on average). A similar response was also observed for several proteins involved in aerobic oxidative phosphorylation, such as components of succinate dehydrogenase (SdhA; at 4070, 5700, 9900 and 18565 min), and ATP synthase (AtpHD; at 4070, 5700 and 9900 min). In addition, the T-profiler analysis revealed a significant increase in overall abundance of proteins involved in oxidative phosphorylation at time 1605 min of the stress (Fig. 5.5).

Taken together, the apparent up-regulation of several proteins involved in the major processes of carbohydrate metabolism and energy generation from 4070 min after applying combined cold and osmotic shifts onward may be to compensate for the reduced activity of these processes under such stress, or may reflect a high level of energy production together with an increase in level of precursors for the biosynthesis of various macromolecules. The latter also agrees well with the period from when these changes became apparent, suggesting that growth might have already been resumed from phase II of the growth curve onward; Fig. 5.1. In support of this, previous studies have also demonstrated an increase in metabolic activities of *E. coli* during exponential growth under the same stress condition (14°C a_w 0.967) (Kocharunchitt et al. 2011).

5.3.2.3 Amino acids biosynthesis

Upon simultaneous cold and osmotic shifts, the present study revealed an immediate decrease in overall abundance of proteins involved in the biosynthesis of the serine family of amino acids with a significant negative *T*-value of 2.10 (Fig. 5.6). This was despite the observation that none of these proteins exhibited a significant down-regulation at this time point (Appendix G.3). Consistent with this finding, Weber and Jung (2002) demonstrated reduced expression of genes (*cysKM*) encoding enzymes responsible for the biosynthesis of cysteine (an amino acid of the serine family) in response to hyperosmotic shift.

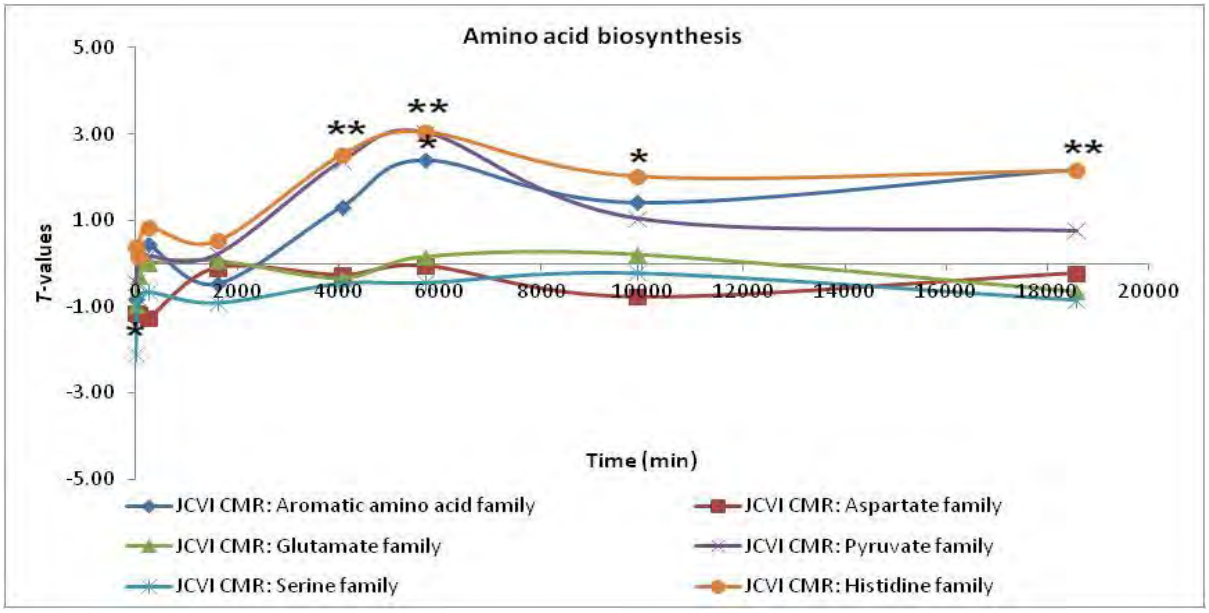

Fig. 5.6. Changes in overall abundance of proteins involved in amino acid biosynthesis during exposure of *E. coli* O157:H7 Sakai to combined cold and osmotic shifts from 35°C a_w 0.993 to 14°C a_w 0.967, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated database, as described in Section 5.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

By contrast, *E. coli* showed increased abundance of a number of proteins for biosynthesis of various amino acids during the period at which growth had occurred after sudden downshifts in temperature and a_w, i.e. from 4070 min of the stress onward (Fig. 5.6). It was found that several enzymes involved in the biosynthesis of pyruvate family amino acids were transiently up-regulated at 4070 and 5700 min of the shifts, as determined by the T-profiler analysis. Among these enzymes, IlvABCD, which are known to have a function in the biosynthesis of isoleucine and valine, exhibited a significant up-regulation. The T-profiler analysis also indicated a significant increase in overall abundance of biosynthetic proteins for aromatic amino acids only at time 5700 and 18565 min. This was despite the observation that many of these proteins were significantly up-regulated from 5700 min of the stress onward, such as those involved in the biosynthesis of chorismate (AroF), tryptophan (AroF and TrpAB), tyrosine (AroF and TyrA) and phenylalanine (AroF and TyrA) (Appendix G.3). Furthermore, a number of proteins involved in histidine biosynthesis were found to be up-regulated with a significant positive *T*-value from 4070 min of the stress onward. These included HisA (at 4070 and 5700 min of the stress), HisC (at time 5700 and 9900 min) and HisDI (from 5700 min onward) with the abundance level of up to 5.40 $\log_2$-fold increase. Taken together, the transient increase in the cellular content of pyruvate family amino acids and accumulation of histidine and aromatic amino acids observed here reflects their importance in mediating growth and/or survival of *E. coli* under combined cold and osmotic stress. This is consistent with previous studies, reporting that increased level of specific amino acids within cells can contribute tolerance to certain stress conditions (Horinouchi et al. 2010; Jozefczuk et al. 2010). In keeping with this, Kocharunchitt et al. (2011) has also revealed the same response of *E. coli* during steady-state growth at 14°C a_w 0.967.

5.3.2.4 Transport systems

A variety of bacteria including *E. coli* are known to accumulate compatible solutes to mediate cellular protection against environmental stresses, including cold and osmotic stresses. This is typically achieved through transport systems to import compounds from the external environment (O'Byrne and Booth 2002; Angelidis and Smith 2003; Ozcan et al. 2005). In the present study, a number of transporters for compatible solutes were found to be significantly up-regulated upon simultaneous cold and osmotic shifts (Appendix G.3). These included the proteins involved in the uptake of glycine betaine/proline (ProVW; from 1605 min of the shifts onward) and osmoprotectants (YehXZ operon; from 5700 min onward). Surprisingly, none of the transport proteins for charged solutes (e.g. potassium and glutamate) were detected in this study. This finding is in contrast to previous studies on the molecular response of *E. coli* to a sudden downshift in temperature (Chapter 3) and a_w (Chapter 4) in which induced production of the low-affinity potassium ion (K^+) transport system (Kup) was observed.

5.3.2.5 Fatty acids and lipids metabolism

It is well established that bacterial growth at low a_w leads to changes in the head group of membrane lipids by increasing the proportion of anionic (PG and CL) over zwitterionic phospholipids (i.e. phosphatidylethanolamine; PE) (Russel et al. 1995; Romantsov et al. 2008). This is in contrast to low temperature in which the major effect on bacterial membrane composition is the fatty acid component of phospholipids. Growth of bacteria at low temperature typically increases the proportion of unsaturated fatty acids in relative to saturated fatty acids, resulting in a lower melting point and a greater degree of flexibility of the lipid membrane (Russel et al. 1995; Yamanaka 1999). Such changes are to maintain the physiological functions

of membrane under the stress conditions of cold temperature and low a_w (Russel et al. 1995). Accordingly, it is expected that changes in cell membrane composition would occur in response to the combination of these stresses. However, the present study revealed that exposure of *E. coli* to combined cold and osmotic stress has no significant effects on overall abundance of proteins involved in the major pathways of fatty acid and lipid metabolism, as indicated by the T-profiler analysis (Fig. 5.7). This was despite the observation that phosphatidylserine decarboxylase (Psd), an enzyme known to be responsible for catalyzing the formation of PE was significantly up-regulated by up to 1.67 $\log_2$-fold from 5700 min after the shifts onward. It was also found that several proteins with functions related to the biosynthesis of fatty acids exhibited a significant increase in abundance in response to combined cold and osmotic stress, including the key enzymes in fatty acid biosynthesis initiation (AccD and FabB; commonly observed at 4070 and 5700 min after the shifts); and elongation of saturated (FabBG; commonly observed at time 5700 min) and unsaturated fatty acids (FabBG; at time 5700 min) (Appendix G.3). Therefore, the apparent lack of abundance patterns of proteins involved in fatty acids and lipids metabolism requires further investigations (e.g. using fatty acid analysis by gas chromatography) to better characterize the cell membrane composition in *E. coli* upon simultaneous cold and osmotic shifts.

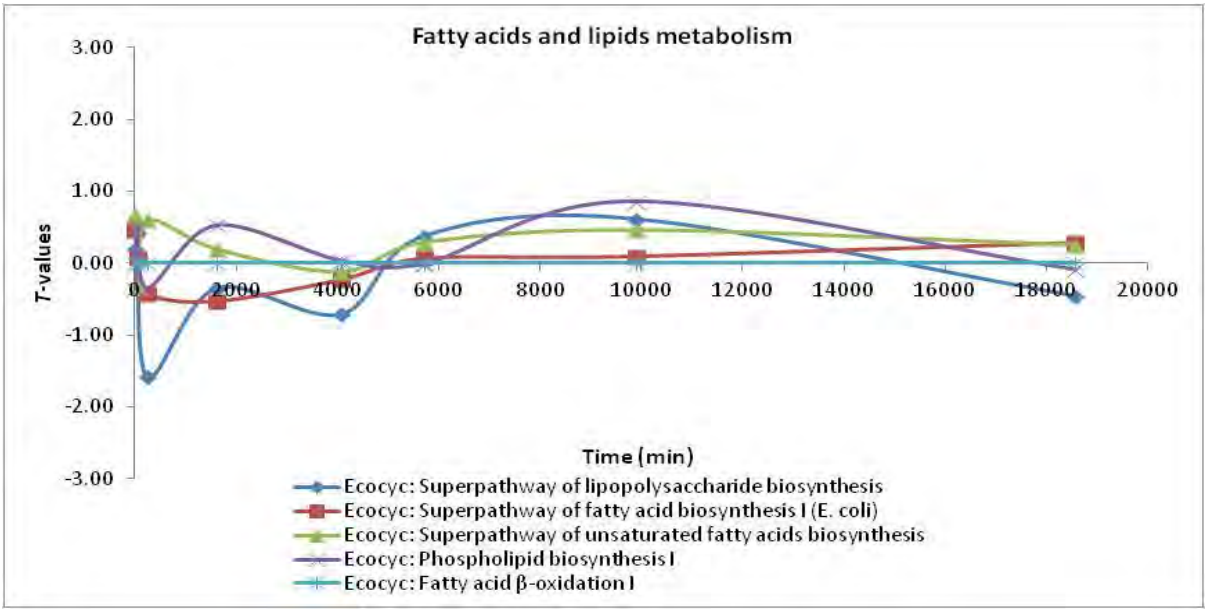

Fig. 5.7. Changes in overall abundance of proteins involved in fatty acids and lipids metabolism during exposure of *E. coli* O157:H7 Sakai to combined cold and osmotic shifts from 35°C a_w 0.993 to 14°C a_w 0.967, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the indicated database, as described in Section 5.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

5.3.2.6 Cell-envelope components

E. coli responded to combined cold and osmotic stress by activating the Rcs phosphorelay system that regulates the biosynthesis of the exopolysaccharide colanic acid (Hagiwara et al. 2003). The T-profiler analysis revealed that several proteins involved in the Rcs regulon exhibited an increase in abundance with a significant positive *T*-value from 250 min after application of the stress onward (Fig. 5.8). These proteins were also amongst the most highly up-regulated in the present study (Appendix G.3). Consistent with this finding, Kocharunchitt et al. (2011) demonstrated that *E. coli* cells strongly up-regulated Rcs-dependent elements and produced the high level of colanic acid during steady-state growth at the condition of combined cold and osmotic stress (14°C a_w 0.967). Colanic acid has been reported to play an important role

in protecting bacterial cells from a variety of environmental stresses, including both low temperature and osmotic stress (Ophir and Gutnick 1994; Chen et al. 2004; Mao et al. 2006), and has been shown to be essential for biofilm formation (Danese et al. 2000). Despite this, the mechanism of stress tolerance conferred by colanic acid remains to be elucidated. Several studies have also suggested that the properties of colanic acid, i.e., to create a physical barrier with a strong negative charge on the outer cell surface, might be one of the contributing factors that mediate the tolerance of cells to stressful conditions (Sledjeski and Gottesman 1996; Chen et al. 2004; Mao et al. 2006). However, it is worth noting that Kocharunchitt et al. (2011) found that colanic acid is not required for growth and survival under combined cold osmotic stress. Further research, therefore, should be conducted to elucidate the physiological function of colanic acid during exposure of bacterial cells to stress conditions.

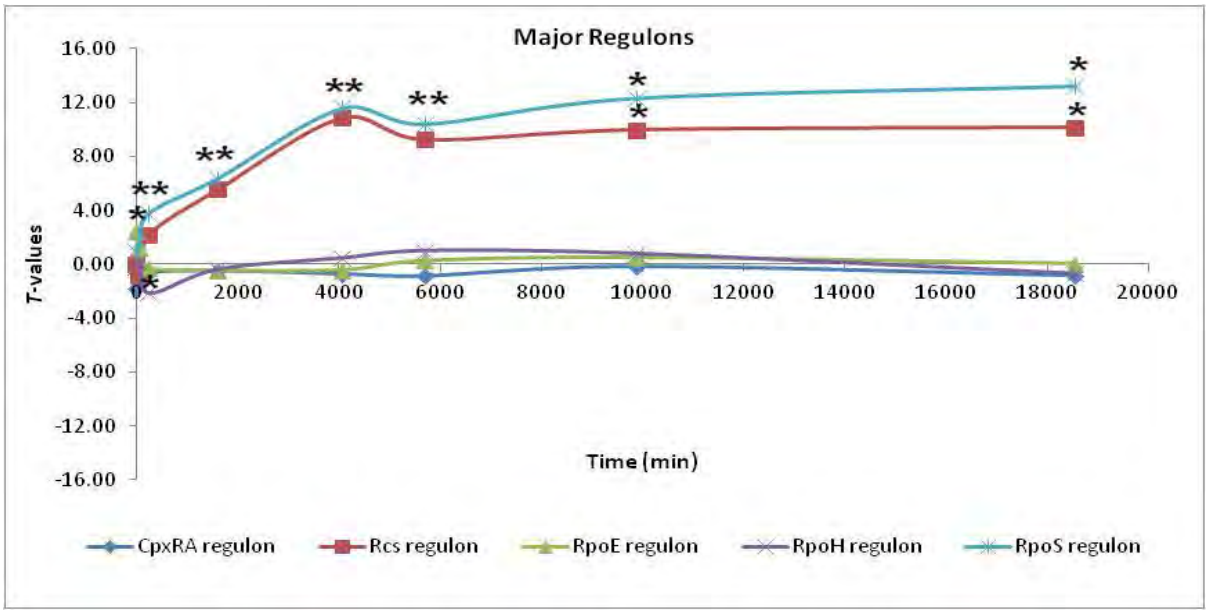

Fig. 5.8. Changes in overall abundance of proteins involved in the major regulons during exposure of *E. coli* O157:H7 Sakai to combined cold and osmotic shifts from 35°C a_w 0.993 to 14°C a_w 0.967, as determined by T-profiler analysis. The predefined sets used for T-profiler analysis were based on the list of genes or proteins previously known to be induced by major regulons, as described in Section 5.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

5.3.2.7 Bacterial chemotaxis and flagellar assembly

The T-profiler analysis revealed a significant increase in overall abundance of proteins involved in chemotaxis and motility within the first 60 min following downshifts in temperature and a_w (Fig. 5.9). In keeping with this, it has previously been demonstrated that osmotic shock alone (from a_w 0.993 to a_w 0.967) but not cold shock alone (from 35°C to 14°C) immediately induced the expression of chemotaxis and motility elements (Chapters 3 and 4). This has led to the hypothesis that up-regulation of proteins involved in chemotaxis and motility observed here may be due to the effects of a sudden downshift in a_w rather than in temperature, and that the presence

of NaCl may stimulate this response. Wecker et al. (2009) also suggests that *E. coli* cells adopt this initial response to move away from the stress condition.

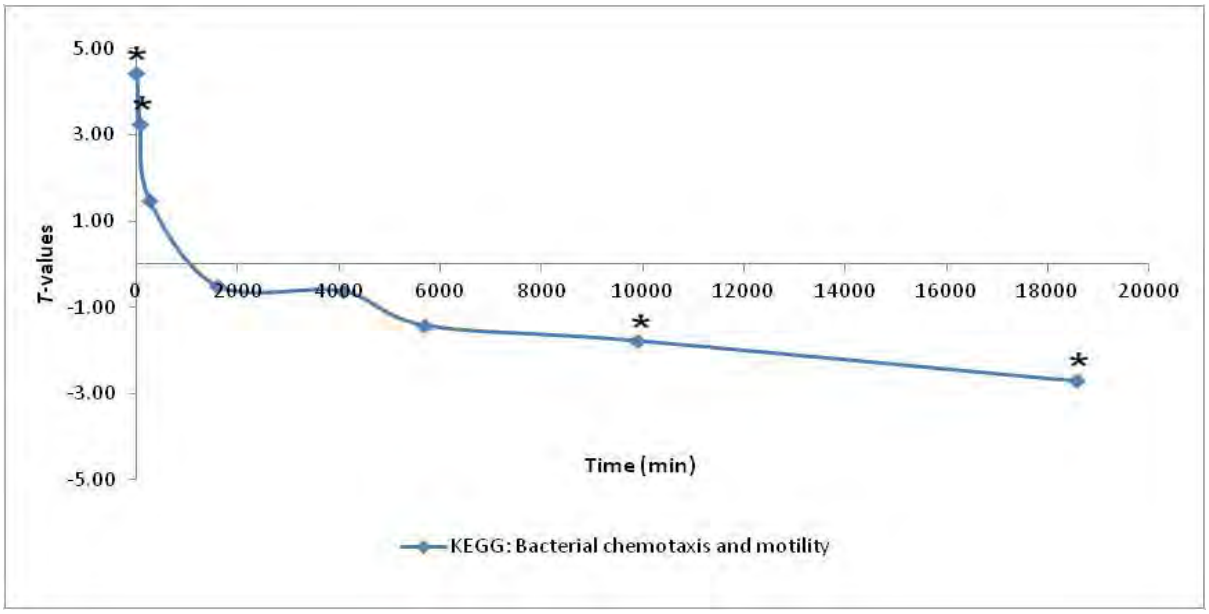

Fig. 5.9. Changes in overall abundance of proteins involved in bacterial chemotaxis and motility during exposure of *E. coli* O157:H7 Sakai to combined cold and osmotic shifts from 35°C a_w 0.993 to 14°C a_w 0.967, as determined by T-profiler analysis. The predefined set used for T-profiler analysis was based on the indicated database, as described in Section 5.2.10. An asterisk indicates significant *T*-value with a *P*-value of <0.1.

On the other hand, the chemotaxis and motility proteins were found to be gradually down-regulated over the course of combined cold and osmotic treatment (Fig. 5.9). This observation agrees well with the study of Kocharunchitt et al. (2011), suggesting that chemotaxis and motility systems are the most dispensable functions during steady-state growth under combined cold and osmotic stress (14°C a_w 0.967). It is also worthwhile noting that the apparent down-regulation of chemotaxis and motility proteins is in line with the fact that expression of genes

encoding flagella are negatively controlled by the master stress regulator RpoS (Patten et al. 2004) and the Rcs system-controlled colanic acid biosynthesis (Francez-Charlot et al. 2003).

5.3.2.8 Time-dependent stress response

The present study employed the K-means clustering algorithm together with principal-component analysis to further analyze a total of 745 proteins that altered their abundance at one or more time points. This approach aims to gain a deeper insight into the abundance pattern of proteins involving the combined cold and osmotic stress response, apart from those already described in major functional groups and metabolic pathways as above. Based on the principal-component analysis, it was found that the protein profile could be explained by five principal components, accounting for approximately 95% of the variance (Fig. 5.10). This means that there were five major independent features required to categorise the time-dependent abundance of each protein (Raychaudhuri et al. 2000). As a consequence, proteins with similar abundance characteristics were assigned into five different clusters (Clusters A-E) by the K-means clustering analysis (Fig. 5.11). The complete list of proteins in each cluster is given in Appendix G.3.

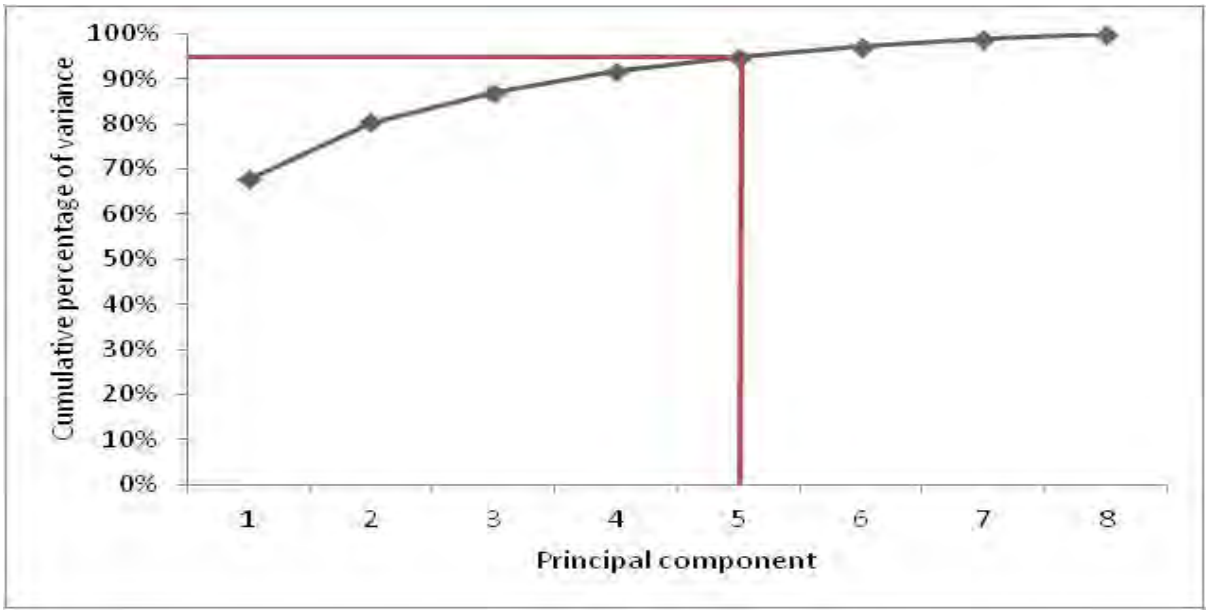

Fig. 5.10. Principal-component analysis of proteins differentially expressed after simultaneous sudden downshifts in temperature and water activity. A scree plot shows the cumulative percentage of variance explained by number of principal components. The first five principal components account for approximately 95% of the variance, as indicated by the solid red line.

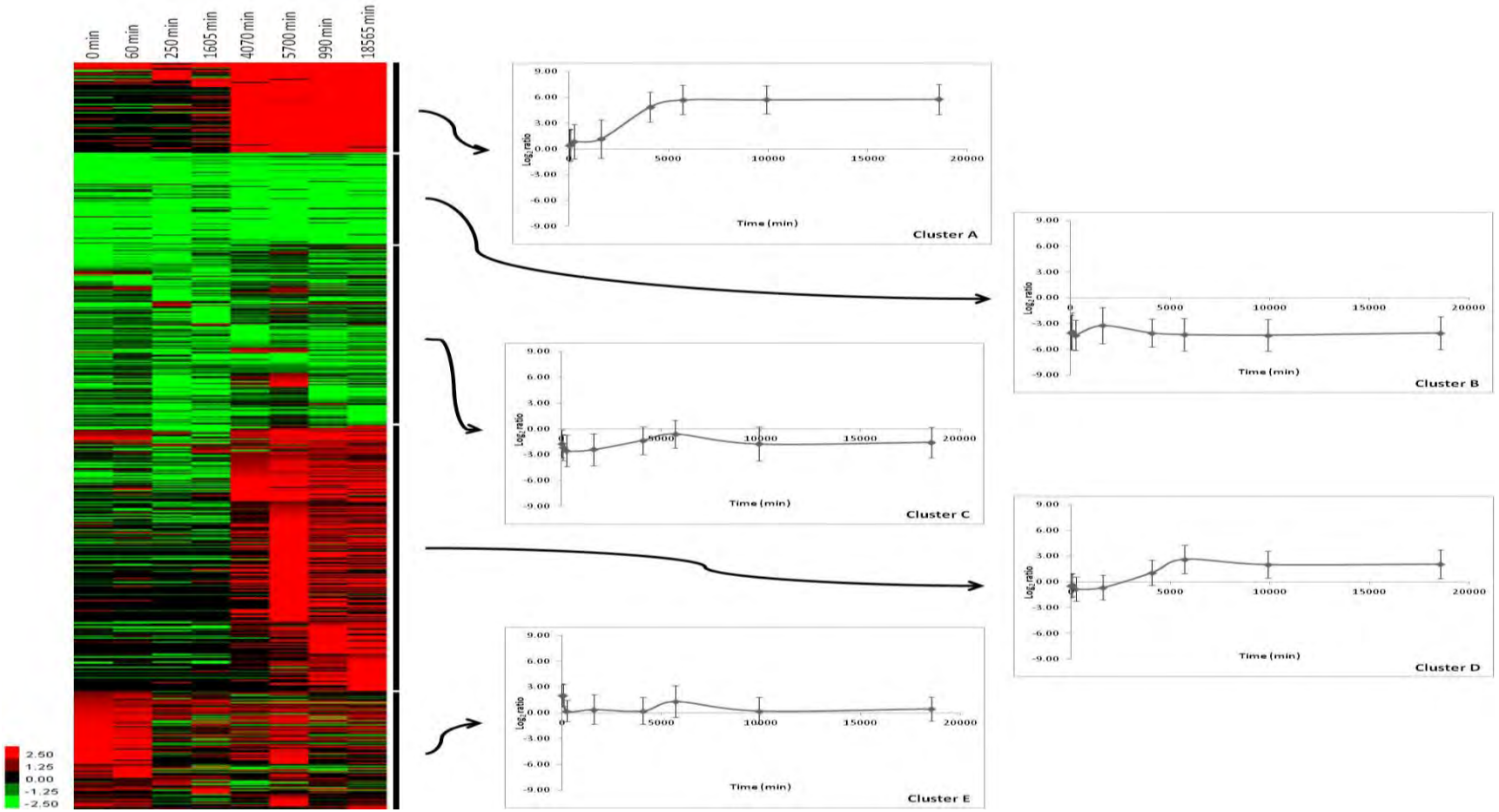

Fig. 5.11. K-means clustering analysis of proteins differentially expressed after simultaneous sudden downshifts in temperature and water activity. Five clusters (A-D) were characterized by K-means clustering according to their specific proteomic profiling. The heat map (left panel) is represented by the abundance levels of proteins (row) for each of the time points (top). Proteins with increased abundance are shown as red, while proteins with decreased abundance are green. The graphs (right panel) represent the average abundance of proteins in each cluster over the time course.

Cluster A

The first cluster represents 94 proteins that exhibited an increase in abundance level at 4070 min after simultaneous cold and osmotic shifts and maintained their level of up-regulation throughout the stress (Fig. 5.11). This abundance pattern corresponds well with the period at which *E. coli* had resumed exponential growth after the shifts (i.e. from phase II of the growth curve onward; Section 5.3.1). This suggests that the proteins in this cluster may be part of adaptive strategies and responses to mediate growth and/or survival of *E. coli* under combined cold and osmotic stress. Indeed, cluster A is mainly composed of proteins with functions related to stress response. These included chaperone proteins (IbpA and Spy), which have functions in facilitating protein folding, and preventing aggregation of misfolded proteins; PspA, which is responsible for maintaining the proton motive force under stress conditions; YncC that regulates biofilm formation and mucoidity; YtfE, which is involved in the repair of damaged iron-sulfur clusters under nitrosative and oxidative stress conditions; and DkgA that is involved in the detoxification of methylglyoxal (a toxic compound produced during glycolysis, fatty acid metabolism and protein metabolism).

It is evident in cluster A that CspG, one of the major cold shock proteins exhibited an increase in abundance during growth under combined cold and osmotic stress. This cold shock protein is thought to have overlapping functions with the well-studied cold shock protein CspA, as an RNA chaperone. Increased production of CspG has also been described to be essential for bacterial cells to resume growth at low temperature (Thieringer and Jones 1998; Yamanaka 1999). However, the abundance pattern of CspG observed here contradicts previous proteomic studies using two-dimensional difference gel electrophoresis (2D-DIGE), which demonstrated that CspG

is up-regulated during acclimation phase upon cold shock, and subsequently down-regulated when cells have become cold-adapted (Thieringer and Jones 1998; Etchegaray and Inouye 1999). This was despite the recent findings that have indicated an up-regulation of *cspG*/CspG in *E. coli* cells at which growth was resumed after a temperature downshift (Chapter 3). It also should be noted that the present study found a transient decrease in abundance of CspA following simultaneous cold and osmotic shifts (i.e. Cluster C). These observations, therefore, suggest that CspG may play more important roles than CspA in response to the stress conditions used in this study. In support of this, Yamanaka (1999) reported that CspG is induced within a narrower range of low temperature than CspA.

E. coli responded to combined cold and osmotic stress by up-regulating the master stress regulator RpoS, and a group of proteins whose transcription is positively regulated by RpoS (Lacour and Landini 2004; Patten et al. 2004; Weber et al. 2005), as indicated by their presence in cluster A. This observation is consistent with the T-profiler analysis, revealing a significant increase in overall abundance of proteins involved in the RpoS regulon from 250 min after application of the combined cold and osmotic stress onward (Fig. 5.8). More specifically, the majority of these RpoS-dependent proteins are involved in osmotic adaptation (Chapter 4; Weber and Jung 2002; Weber et al. 2006; Moen et al. 2009). These included osmotically inducible proteins (OsmBCEY); Dps, which is responsible for the protection of DNA; OtsAB, which are involved in the *de novo* synthesis of trehalose; TreF, which controls the intracellular level of trehalose; YhbO, which regulates RNA/protein interaction, signal transduction, thiamine biosynthesis and proteases; and a group of proteins with undefined functions (EleB, YciEF, YgaM and YjbJ). Other proteins of the RpoS regulon in this cluster are also identified, and

appear to be associated with response to oxidative damage, such as KatE and SufACS. In keeping with these, Kocharunchitt et al. (2011) has demonstrated activation of the master stress regulator RpoS during exponential growth of *E. coli* under the same stress condition (14°C a_w 0.967). The present findings also agree well with previous studies, reporting that the general response network established by the RpoS regulon typically provides cross-protection against diverse stress conditions (Lacour and Landini 2004; Patten et al. 2004; Weber et al. 2005; Kocharunchitt et al. 2011). Furthermore, it has previously been demonstrated that prolonged exposure of *E. coli* cells to cold stress (14°C) resulted in down-regulation of several elements involved in response to oxidative stress (Chapter 3), whereas expression of these elements was induced during growth at hyperosmolality (a_w 0.967) (Chapter 4). This has led to the suggestion that the increase in abundance of oxidative stress-responsive proteins observed here may be due to the effects of osmotic stress rather than cold stress. However, Kocharunchitt et al. (2011) reported up-regulation of several genes involved in oxidative stress resistance in *E. coli* cells grown under the conditions of low temperature (14°C a_w 0.985) and a_w stress (25°C a_w 0.967).

Cluster B

Cluster B consists of 90 proteins that were down-regulated during the whole period of exposure to combined cold and osmotic stress (Fig. 5.11). It is suggested that proteins with this abundance pattern may not be required for cells to resume growth and survive under the stress condition. Equally, decrease in synthesis of these proteins may be to prevent an inappropriate use of cellular energy. Cluster B is formed by a wide range of proteins with diverse functions (Appendix G.3). Notably, these included a group of proteins (Rnc and Rnd), which is responsible for RNA degradation; TrmABJ, which have functions in tRNA and rRNA base modification; SdaB that is

a key enzyme in the pathway of serine degradation; and WbdOQ, which are putative enzymes involved in the biosynthesis of lipopolysaccharide.

Cluster C

Cluster C is composed of 183 proteins whose abundance was transiently reduced within the first 1605 min of combined cold and osmotic stress (Fig. 5.11). Such abundance characteristic of proteins is consistent with the period at which growth arrest or adaptation phase was observed upon simultaneous cold and osmotic shifts (Section 5.3.1). This indicates that the functions of these proteins may contribute to the prolonged growth arrest of *E. coli* after the shifts. Among proteins in cluster C, a number of proteins involved in defense mechanisms against oxidative damage are present, including alkylhydroperoxide reductase (AhpCF), Hydroperoxidase I (KatG), and lipid hydroperoxide peroxidase (Tpx). This finding is consistent with the study of Mackey and Derrick (1986), demonstrating that *E. coli* became sensitive to oxidative stress upon cold shock. It has also been shown that several oxidative stress-responsive elements were down-regulated during growth arrest upon a temperature downshift (from 35°C to 14°C) (Chapter 3), whereas no obvious trend of response was observed for expression of these elements following hyperosmotic shock (from a_w 0.993 to a_w 0.967) (Chapter 4).This indicates that the apparent down-regulation of proteins involved in oxidative stress response may be related to the effects of cold shock rather than osmotic shock.

Exposure of *E. coli* to sudden downshifts in temperature and a_w resulted in a transient down-regulation of several chaperones (ClpX, DnaK, GrpE and HtpG), as indicated by their presence in cluster C. All of these chaperones have previously been described to be involved in the RpoH

regulon that is known to be responsible for the control of protein misfolding in the cytoplasm (Alba and Gross 2004; Nonaka et al. 2006). Accordingly, the T-profiler analysis revealed a significant decrease in overall abundance of proteins involved in protein folding and stabilization within the first 250 min of the stress and those proteins involved in the RpoH regulon at time 250 min (Fig. 5.8). This was despite the observation that RpoH protein did not exhibit a significant change in abundance. The apparent down-regulation of chaperone proteins during growth arrest indicates that *E. coli* cells may lose their efficiency in repairing protein misfolding and facilitating the proper folding of newly synthesized proteins following combined cold and osmotic shifts. In keeping with this, the recent findings of Strocchi et al. (2006) have indicated that the growth arrest of *E. coli* upon cold shock appeared to be associated with inactivation of the chaperone system. It has also been shown previously that a sudden downshift in temperature resulted in down-regulation of several elements involved in protein folding machinery (Chapter 3; Kim et al. 2005). Additionally, it is worthwhile noting that other chaperone proteins of the RpoH regulon were observed in this study. Many of these (DnaJ, GroEL and HtpX) appeared to be up-regulated within the first 60 min of combined cold and osmotic stress (i.e. Cluster E).

Cluster D

Among the five clusters, cluster D forms the largest group and contains 260 proteins. These proteins were found to have a similar pattern of abundance profiles to those of cluster A (i.e., increased abundance after 4070 min of combined cold and osmotic shocks onward), although their magnitude of induction was relatively weaker (Fig. 5.11). It is, therefore, suggested that the proteins in this cluster may also play an important role in promoting growth and/or survival under combined cold and osmotic stress. Similar to cluster A, cluster D comprises several

proteins previously known to be induced by RpoS at their transcriptional level (Lacour and Landini 2004; Patten et al. 2004; Weber et al. 2005) (Appendix G.3). This was despite that these RpoS-dependent proteins are mainly associated with response to other stresses (rather than osmotic stress in cluster A), such as acid (GadAB, HdhA and Slp) and oxidative damage (SodC and SufB). A number of other proteins involved in adaptation to stress conditions are also present in cluster D. These included putative catalase (ECs1652); universal stress proteins (UspAE); and YieF that possesses quinone reductase activity, providing cellular protection against oxidative stress.

It was observed in cluster D that there are several proteins involved in the DNA repair system, such as a component of the RecFOR complex (RecR) that functions in RecA-mediated replication recovery; RecA, which serves as a regulatory protein to induce the SOS response to DNA damage; and subunits of the UvrABC nucleotide excision repair complex (UvrAB). An increase in abundance of these DNA repair enzymes might indicate that DNA damage occurs upon exposure to sudden downshifts in temperature and a_w, and that repairing the damaged DNA is required to establish growth following the shifts. In line with this, previous studies have reported that DNA damage resulted from osmotic shock (Fitt et al. 1992; Ribeiro et al. 2006) and cold shock (Ray 2006). It also should be noted that previous findings of this thesis did not provide strong evidence, suggesting induced activity of the DNA repair system in either cold-adapted cells (Chapter 3) or osmotically-adapted cells (Chapter 4).

E. coli up-regulated a number of proteases during the period at which growth was re-established after simultaneous cold and osmotic shifts, as indicated by their presence in cluster D. These

included proteins known to be responsible for the degradation of proteins (ClpAP and Lon) and peptides (Dcp, PrlC and HslU), which are, respectively, important for processing irreversibly damaged or denatured proteins and peptides. The increase in abundance of proteases observed here may be a response for *E. coli* to cope with the increased levels of protein misfolding and aggregation caused by the down-regulation of chaperones (see Section "Cluster C") in order to re-establish growth under combined cold and osmotic stress. This agrees well with the recent study on the response of *Pseudomonas putida* during growth at low temperature (Fonseca et al. 2011). However, it should be noted that the previous study on the molecular response of *E. coli* to a temperature downshift did not observe this expression pattern of genes and proteins in cold-adapted cells (Chapter 3).

Another group of interesting proteins in cluster D is those with functions related to cell division. These were components of the *min* system (MinDE) that is responsible for the correct placement of the division site (the Z-ring septum) at mid-cell; MreB, which functions in proper chromosome segregation; essential cell division proteins (FtsAI); and ZapA, which promotes the formation of the Z-ring. The abundance characteristic of these cell division-related proteins observed indicates that growth has been established from 4070 min of combined cold and osmotic treatment onward. This further supports the hypothesis derived from the growth study that *E. coli* might have already resumed growth from phase II of the growth curve onward, although the physiological basis of the subsequent period, in which bacterial numbers were unchanged, remains to be elucidated (i.e. during phase III; see Section 5.3.1).

Cluster E

Cluster E represents 118 proteins whose abundance was transiently induced within the first 60 min after simultaneous cold and osmotic shifts (Fig. 5.11). This abundance characteristic of proteins indicates that their physiological functions are part of the initial response of *E. coli* to the shifts (i.e. an 'emergency' response). Specifically, many proteins of this cluster have previously been shown to be involved in the RpoE regulon that controls protein misfolding in the cell envelope (Alba and Gross 2004; Nonaka et al. 2006; Rhodius et al. 2006). These included RseC, which is a positive regulator of RpoE activity; outer-membrane protein OmpX; PqiB whose transcription is induced in response to superoxide generators; Imp, which has functions in the proper assembly of lipopolysaccharides at the surface of the outer membrane; YaeT that is responsible for outer membrane protein biogenesis; and a protein with undefined functions (YjeP), despite that RpoE was not detected in the present study. Similarly, the T-profiler analysis also revealed a significant induction of the RpoE regulon immediately after the shifts (Fig. 5.8). Activation of this regulon probably serves as a multipurpose emergency response to attempt to repair protein misfolding at the cell surface. The apparent response of *E. coli* to combined cold and osmotic shifts through a transient induction of the RpoE regluon is also in keeping with previous studies on the cellular response of *E. coli* to an abrupt downshift in temperature (Chapter 3; Polissi et al. 2003) and a_w (Chapter 4; Bianchi and Baneyx 1999).

Upon simultaneous cold and osmotic shifts, *E. coli* transiently increased abundance of several proteins associated with the transport system for metallic cation, iron-siderophore and vitamin B12, as evident by their presence in cluster E. A component of the TonB-ExbBD energy transducing system (ExbB) that provides the energy source required for the uptake of iron-

siderophore complexes and vitamin B12 across the outer membrane was observed. This was accompanied by the presence of FhuACD and FepA, which are respectively subunits of the TonB-dependent uptake system for iron (III) hydroxamate and ferric enterobactin. CirA and Fiu that act as receptors for siderophores and colicin are also grouped in this cluster. It has previously been reported that the induced activity of iron uptake systems is strongly linked with an increase in the cellular levels of hydrogen peroxide (H_2O_2) and superoxide (O_2^-). The high levels of these reactive oxygen species (ROS) can induce the oxidative stress response (Horinouchi et al. 2010). Therefore, the apparent enhancement of iron uptake indicates that *E. coli* cells may suffer from oxidative damage in addition to combined cold and osmotic stress. This probably explains the present observation in which a number of oxidative stress-responsive proteins exhibited an up-regulation during growth under the stress condition (see Sections "Cluster A" and "Cluster D"). Furthermore, a wide range of other transport systems were found to be members of this cluster. These included those proteins involved in the uptake of lipoprotein (LolCE), methionine (MetIN), and nucleoside (Tsx).

5.3.2.9 Overall stress response

Taken together, the present data suggest a spectrum of time-dependent changes in the physiological response of *E. coli* to simultaneous sudden downshifts in temperature and water activity, as summarized in Fig. 5.12.

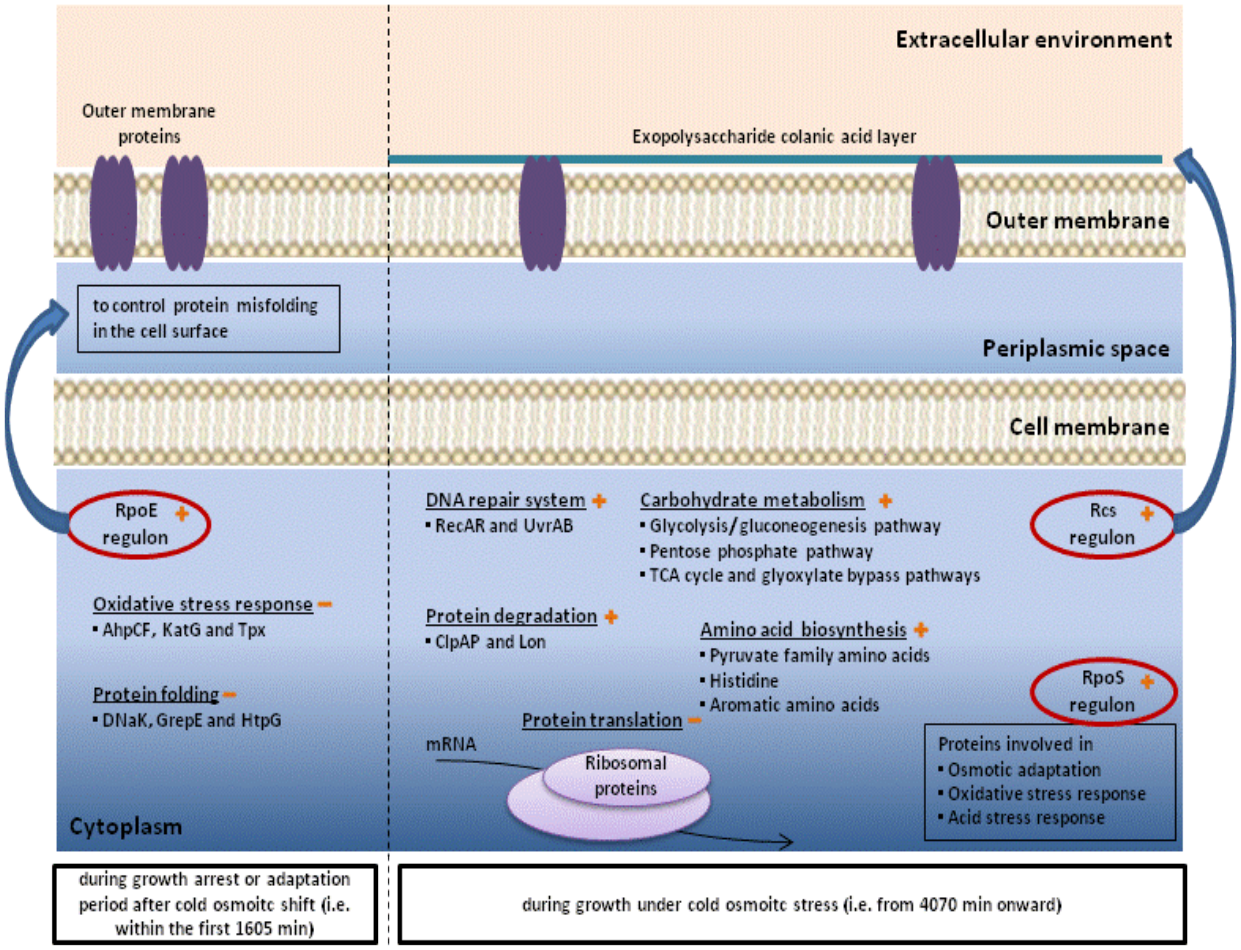

Fig. 5.12. A model for proteomic response of *E. coli* O157:H7 Sakai to simultaneous sudden downshifts in temperature and water activity from 35°C a_w 0.993 to 14°C a_w 0.967. The increased and decreased abundance is represented by + and -, respectively.

5.4 CONCLUDING REMARKS

This pioneering investigation was undertaken to provide a detailed understanding of the growth kinetics and proteomic response of exponentially growing *E. coli* O157:H7 Sakai subjected to simultaneous sudden downshifts in temperature and water activity (i.e. from 35°C a_w 0.993 to 14°C a_w 0.967). Exposure of *E. coli* to combined cold and osmotic shifts resulted in a complex pattern of microbial population changes. This growth behavior can be divided into three successive phases: (i) an initial decline in bacterial numbers followed by a period in which

numbers increased rapidly; (ii) a second decrease in culturable cells and subsequent 'exponential-like' growth at a rate similar to that expected under the conditions; and (iii) a constant population level, similar to the starting density before imposition of the combined shocks (i.e. analogous to a 'lag' phase), until 'true exponential' growth was (re)-established. Of particular interest, analysis of kinetic parameters for each phase revealed that the GT estimate for phase I was shorter than those of phases II and III. This suggests an apparent loss, then recovery, of cell culturability occurred during the first phase. In contrast, there were no differences in the GTs between phases II and III, suggesting that *E. coli* may have already resumed exponential growth from phase II onward. Other parameters such as AT and RLT were found to increase when determined at later and later phases in the growth curve.

In proteomic analysis, *E. coli* responded to simultaneous cold and osmotic shifts by altering abundance levels of proteins involved in several functional groups and metabolic pathways. A number of these changes were found to mediate different stages of adaptation in relation to dynamic changes in growth kinetics as occurred during exposure to the shifts. Notably, the RpoE regulon was activated during the initial loss and subsequent recovery of culturability (phase I). This implies its critical role as an emergency response for cells to repair protein misfolding in the cell envelope, possibly enabling them to survive from combined cold and osmotic shifts. The apparent down-regulation of molecular chaperones and those proteins associated with responses to oxidative damage during growth arrest or adaptation phase may be one of the contributing factors to the prolonged growth arrest of *E. coli* after the shifts. Finally, growth under combined cold and osmotic stress was found to activate the master stress regulator RpoS and the Rcs system-controlled colanic acid biosynthesis, as well as to increase abundance of several proteins

with diverse functions including those involved in the DNA repair system, the degradation of proteins and peptides, the amino acid biosynthetic pathways and the major processes of carbohydrate catabolism and energy generation (i.e. the glycolysis/gluconeogenesis pathway, the pentose phosphate pathway and the TCA cycle and glyoxylate bypass pathways). This reflects their important functions in establishing and promoting growth and/or survival under such stress. The results presented here will form knowledge foundation with genome-wide studies (e.g. cDNA microarray analysis) to develop a full understanding of the functional architecture of genomes and gene networks of *E. coli* that may aid the development of novel approaches for the meat industry to combat this pathogen.

CHAPTER 6

FINAL DISCUSSION AND SUMMARY

The primary objective of this thesis was to gain a comprehensive understanding of the physiological responses of *E. coli* O157:H7 strain Sakai subjected to conditions relevant to low temperature and water activity experienced during meat carcass chilling in cold air. This was initially approached by investigating the physiology of *E. coli* during balanced growth under cold temperature and water activity stress. A series of studies was then undertaken to provide insight into the potential mechanisms enabling survival and growth of *E. coli* upon exposure to dynamic low temperature and water activity conditions.

In the present research, both transcriptomic (cDNA microarray) and proteomic (2D-LC/MS/MS) analyses were used to characterize the physiological responses of *E. coli*. These analyses have proven to be a useful tool for the study of an entire collection of mRNA transcripts and protein abundances that provide the description of complex biological systems within the cells (Vollmer et al. 2003; Weber et al. 2005; Allen et al. 2008; Ishihama et al. 2008). However, several studies have reported conflicting evidence regarding the correlation between large-scale transcriptomic and proteomic datasets (Ideker et al. 2001; Chen et al. 2002; Griffin et al. 2002; Tian et al. 2004). This is consistent with the present observation in which the level of correlation between gene expression and protein abundance profiles appeared to be weak (i.e. approximately less than 12% of genes and proteins matched; see Sections 2.3.1, 3.3.2 and 4.3.2). Several possible explanations for this have been reported, including different controlling mechanisms at the transcriptome and proteome levels, posttranscriptional mechanisms affecting the protein translation rate,

differences occurring between the half-lives of specific mRNAs or proteins, and the intracellular location and molecular association of the protein products of expressed genes (Gygi et al. 1999). It has also been reported that the transcriptomic dataset at a particular time point should be more closely related with proteomic data that are generated at a later time point, i.e. after the processes of translation and post-translational modification has responded to the earlier changes in transcription levels (Rossouw et al. 2010). Despite this, complementary profiling of transcriptome and proteome are still required to develop a full understanding of the functional architecture of genomes and gene networks (Hatzimanikatis et al. 1999; Hatzimanikatis and Lee 1999).

During the initial study, the global expression of *E. coli* under steady-state conditions of cold temperature and water activity stress was elucidated (Chapter 2). *E. coli* universally responded to all conditions of chill temperature and/or low water activity by increasing the expression of genes and proteins controlled by the RpoS general stress response regulon and the Rcs regulon involved in colanic acid biosynthesis, as well as down-regulating the elements involved in chemotaxis and motility. However, colanic acid-deficient mutants were shown to achieve comparable growth rates to their wild-type parents under all conditions, indicating that colanic acid is not required for growth. These results provided a baseline of knowledge of the physiology of *E. coli* to better interpret, and potentially exploit, its responses to dynamic environmental conditions that occur during carcass chilling.

A set of investigations were carried out to characterize the growth kinetics and time-dependent changes in genome and proteome profiles of *E. coli* subjected to a sudden downshift in

temperature (Chapter 3) and water activity (Chapter 4). Shifting *E. coli* to low temperature (below 25°C) caused a lag period of growth before resumption of growth at a rate typical of the temperature downshift experienced. Both generation and lag times increased with the magnitude of the shift, while a similar amount of *work* (i.e. relative lag time) required to adapt to each temperature tested was observed. On the other hand, *E. coli* exposed to low water activity (below a_w 0.985) resulted in an apparent loss, then recovery, of culturability before the growth was resumed. This recovery also occurred at a rate unexpectedly faster than that of exponential growth under a given stress condition. Estimates of kinetic parameters, including generation, lag, and adaptation times were found to become progressively larger with respect to the magnitude of the shift. However, relative lag times as determined by dividing lag time or adaptation time with generation time appeared to be different. $RLTs_{LT/GT}$ increased with larger shifts in a_w, whereas $RLTs_{AT/GT}$ displayed little variation across the a_w range tested.

From transcriptomic and proteomic analysis, exposure of *E. coli* to cold shock (from 35°C to 14°C) and hyperosmotic shock (from a_w 0.993 to a_w 0.967) evoked a highly complex regulatory process involving expression changes of different groups of genes and proteins. A number of these genes and proteins are part of well-established adaptive responses to both cold and osmotic stresses, including those elements involved in accumulation of compatible solutes. *E. coli* also altered expression of other elements, which are associated with several functional groups and metabolic pathways, to contribute to different stages of adaptation to both stresses. Notably, induction of a number of genes and proteins with the cell envelope-related functions (i.e. the biosynthesis of the exopolysaccharide colanic acid) was observed. This implies that cells undergo remodeling of their envelope composition, enabling them to survive the stress better.

Growth at cold temperature and low water activity, however, appeared to up-regulate additional elements, which are involved in the biosynthesis of specific amino acids. This suggests their important role in facilitating growth and/or survival under such stress. On the other hand, it was found that the chemotaxis and motility systems were heavily down-regulated, indicating these functions to be most dispensable during exposure to cold temperature and water activity stress. Finally, the findings of such studies revealed the remarkable adaptability of *E. coli* to survive under dynamic stress condition through activation of multiple stress responses. These responses were mediated by transiently inducing the activity of RpoE regulon to repair protein misfolding and aid the proper folding of newly synthesized proteins in cell envelope, while simultaneously activating the master stress regulator RpoS to mediate long-term adaptation under the stress conditions of low temperature and water activity.

In the final study (Chapter 5), population kinetic responses and cellular physiological responses of *E. coli* after simultaneous abrupt downshifts in temperature and water activity (i.e. from 35°C a_w 0.993 to 14°C a_w 0.967) were assessed. *E. coli* exposed to both cold temperature and low water activity was found to have a complex growth response, which could be divided into three successive phases. The first phase is comprised of an initial decline in cell numbers followed by a period in which numbers increased rapidly, while the secondary decrease in bacterial populations and subsequent 'exponential-like' growth occurred during the second phase. Finally, the third phase is when the populations appeared to be constant at a similar level to the starting numbers (i.e. analogous to a 'lag' phase) until 'true exponential' growth was (re)-established. In an attempt to explain the complexity of the growth response of *E. coli* to combined cold and osmotic shocks, analysis of kinetic parameters for each phase revealed that the generation time

estimated for phase I was shorter than those of phases II and III. This indicates that an apparent loss, then recovery, of cell culturability occurred during the first phase. However, there were no differences in the generation times between phases II and III, suggesting that *E. coli* may have already resumed exponential growth from phase II onward.

Characterization of the protein abundance profiles of *E. coli* upon simultaneous cold and osmotic shifts provided evidence for the mechanisms mediating adaptation under such stress condition. Specifically, the RpoE regulon was activated during the initial loss and subsequent recovery of culturability (phase I). This implies its critical role as an emergency response for cells to repair protein misfolding in the cell envelope, possibly allowing them to recover and survive from injury as a result of the shifts. However, *E. coli* appeared to re-establish growth and survive under combined cold and osmotic stress by activating the master stress regulator RpoS and the Rcs system-controlled colanic acid biosynthesis, as well as increasing abundance of several proteins with diverse functions including those involved in the DNA repair system, the degradation of proteins and peptides, the biosynthetic pathways of specific amino acids and the major processes of carbohydrate catabolism and energy generation.

Despite that an integrated transcriptomic and proteomic analysis employed in the current research provides a comprehensive insight into the physiological response of *E. coli* to dynamic changes experienced during carcass chilling, there are still various aspects required for further investigation. Firstly, it is important to examine further the physiology of the changes occurring to *E. coli* cells during the initial loss and subsequent recovery of culturability after simultaneous abrupt downshifts in temperature and water activity. The study could involve the use of different

fluorescence dyes together with flow cytometry to investigate the morphological and physiological changes that occur. These dyes may include those that are indicative for membrane integrity (e.g. propidium iodide), membrane potential (e.g. 3,3'-dihexyloxacarbocyanine iodide), intracellular pH (e.g. 5(6)-carboxyfluorescein diacetate succinimidly ester), enzyme activity (e.g. carboxyfluorescein diacetate), and generation of superoxide radical (e.g. hydroethidine). The second aspect for future research would be to carry out a metabolic screen (i.e. metabolomic analysis) to ascertain the substrates and products being utilized and/or produced by *E. coli* cells during exposure to combined cold and osmotic shifts. This would provide a more in-depth understanding of *E. coli* physiology. It would also be interesting to determine if the metabolomic profiles correlate well with transcriptomic and/or proteomic profiles generated here. Finally, a series of further experiments are required to test the hypotheses derived from the current research. One such experiment may be conducted to evaluate the potential for using oxidizing agents (e.g. hydrogen peroxide) to eliminate *E. coli* cells when subjected to simultaneous cold and osmotic shifts. This will involve studies of the sensitivity of *E. coli* to these oxidizing agents at different stages during exposure to the shifts. The results obtained would help to develop additional interventions that might be more effective in eliminating or controlling this organism on carcasses.

In conclusion, this thesis has contributed toward understanding the physiological responses of *E. coli* to dynamic environmental conditions that occur during carcass chilling under Australian conditions. Such knowledge will aid the development of more targeted, and less invasive approaches (e.g. the application of additional stresses such as oxidative stress), for the meat industry to eliminate or control this pathogen.

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APPENDIX A

PREPARATION OF BACTERIOLOGICAL MEDIA AND REAGENTS

A.1 100 mM Calcium chloride solution

Anhydrous calcium chloride (Sigma-Aldrich, Australia)	0.27 g
Deionised water (18.2 mΩ)	25 ml

Dissolve calcium chloride in deionised water. Autoclave at 121°C for 20 min.

A.2 400 mM Ammonium bicarbonate stock

Ammonium bicarbonate (Sigma-Aldrich, Australia)	1 g
Deionised water (18.2 mΩ)	25 ml

Dissolve ammonium bicarbonate in deionised water. Autoclave at 121°C for 20 min.

A.3 8 M Urea, 100 mM ammonium bicarbonate

Urea (Sigma-Aldrich, Australia)	2.7 g
400 mM Ammonium bicarbonate stock solution (Appendix A.1.2)	1.25 ml
Deionised water (18.2 mΩ)	≈4 ml

Dissolve urea in the ammonium bicarbonate stock. Adjust the volume 5 ml with deionised water.

Autoclave at 121°C for 20 min.

A.4 Alkylating reagent (200 mM iodoacetamide, 100 mM ammonium bicarbonate)

Iodoacetamide	36 mg
400 mM Ammonium bicarbonate stock solution (Appendix A.1.2)	250 µl
Deionised water (18.2 mΩ)	750 µl

Dissolve iodoacetamide in the ammonium bicarbonate stock. Add deionised water. Autoclave at 121°C for 20 min.

A.5 Brain-hear infusion (BHI) agar

Brain-hear infusion broth (CM225, Oxoid, Australia)	37 g
Agar	15 g
dH_2O	1000 ml

Autoclave at 121°C for 20 min.

A.6 Brain-hear infusion (BHI) agar with 0.1% sodium pyruvate

Brain-hear infusion broth (CM225, Oxoid, Australia)	37 g
Sodium pyruvate (P8574, Sigma, USA)	1 g
Agar	15 g
dH_2O	1000 ml

Autoclave at 121°C for 20 min.

A.7 Brain-hear infusion (BHI) broth

Brain-hear infusion broth	37 g
dH_2O	1000 ml

Autoclave at 121°C for 20 min.

A.8 Digestion buffer (50 mM Ammonium bicarbonate, 1 mM calcium chloride solution)

400 mM Ammonium bicarbonate solution (Appendix A.1.2)	500 µl
Deionised water (18.2 mΩ)	3.45 ml
100 mM Calcium chloride solution (Appendix A.1.1)	40 µl

Mix the ammonium bicarbonate solution with deionised water. Add calcium chloride solution.

Autoclave at 121°C for 20 min.

A.9 Peptone water (PW)

Bacteriological peptone (LP0037, Oxoid, Australia)	1 g
dH_2O	1000 ml

Autoclave at 121°C for 20 min.

A.10 Phosphate buffered saline (PBS)

Phosphate buffered saline (BR0014G, Oxoid, Australia)	1 tablet
dH_2O	100 ml

Autoclave at 121°C for 20 min.

A.11 Reducing reagent (50 mM dithiothreitol, 100 mM ammonium bicarbonate)

Dithiothreitol	7.5 mg
400 mM Ammonium bicarbonate stock solution (Appendix A.1.2)	250 µl
Deionised water (18.2 mΩ)	750 µl

Dissolve dithiothreitol in the ammonium bicarbonate stock. Add deionised water. Autoclave at 121°C for 20 min.

APPENDIX B

DETAILED DESCRIPTION OF THE 2D-LC/MS/MS ANALYSIS

All tryptic digests were analyzed by MudPIT as described previously (Delahunty and YatesIII 2005). In this study, a nanoflow triphasic MudPIT system were used, which consisted of a C18 capillary trap (Peptide CapTrap, Michrom BioResources, Auburn, CA), a strong cation exchange (SCX) material (IntegraFrit Column, 100 μm i.d., 2.5 cm, New Objective, Woburn, MA), and an analytical C18 nano-column (PicoFrit Column, 15 μm i.d. pulled tip, 10 cm, New Objective). This triphasic microcapillary column was directly coupled in-line with an electrospray ionization (ESI) ion trap tandem mass spectrometer (LTQ Orbitrap, ThermoElectron, San Jose, CA).

Typically, a total of 50 μl of each tryptic-digested sample was loaded onto a C18 capillary trap at a flow rate of 35 μl/min using 0.1 % formic acid, 0.1% triflouroacetic acid. During the first 10 min of sample loading, the SCX and analytical columns were switched via a valve out of line of the C18 capillary trap, with the trap being washed to waste to ensure that salts, any excess peptides and other non-peptide materials were not introduced into the mass spectrometer. Thereafter, the capillary trap was placed in-line with SCX and analytical columns, and the flow rate was reduced via a splitter to 250 nl/min. A 5-step chromatography run was carried out on each sample as described in Table S1. The three buffer solutions used for the chromatography were 5% acetonitrile/0.1% formic acid (buffer A), 90% acetonitrile/0.1% formic acid (buffer B), and 500 mM ammonium acetate/5% acetonitrile (buffer C). Additionally, a 1.5-h cleanup step was performed to ensure no sample contamination between runs occurred (Table B.1).

Table B.1 A 5-step MudPIT gradient program and column cleanup step.

Step	Time (min)	%A	%B	%C
1	0	100	0	0
	5	100	0	0
	10	85	15	0
	70	55	45	0
	80	0	100	0
	90	0	100	0
	90.1	100	0	0
	95	100	0	0
2	0	90	0	10
	5	90	0	10
	5.1	100	0	0
	20	85	15	0
	75	75	25	0
	125	50	50	0
	125.1	100	0	0
	130	100	0	0
3	0	80	0	20
	5	80	0	20
	5.1	100	0	0
	20	85	15	0
	75	75	25	0
	125	50	50	0
	125.1	100	0	0
	130	100	0	0
4	0	60	0	40
	5	60	0	40
	5.1	100	0	0
	20	85	15	0
	75	75	25	0
	125	50	50	0
	125.1	100	0	0
	130	100	0	0
5	0	0	0	100
	15	0	0	100
	15.1	100	0	0
	25	80	20	0
	90	55	45	0
	95	0	100	0
	115	0	100	0
	115.1	100	0	0
	130	100	0	0
Cleaning	0	0	100	0
	10	0	100	0
	11	0	0	100
	19	0	0	100
	20	100	0	0
	50	0	100	0
	75	0	100	0
	76	100	0	0
	90	100	0	0

During the MudPIT analysis, the peptides eluted from the microcapillary column were electrosprayed directly into the LTQ ion trap mass spectrometer with the application of a distal 2-kV spray voltage. The mass spectrometer was operated in the data-dependant mode to automatically switch between MS and MS/MS acquisition. A 'survey' scan was performed in the electrostatic Orbitrap to identify the possible parent peptide masses (from m/z 460 to 2,000 Da with the resolution set to 30,000), and the eight most intense peaks were selected and fragmented in the ion trap, producing MS/MS data. This process was performed for 2 s, and was cycled continually throughout the MudPIT run. This typical 2D-LC/MS/MS analysis produced between 10,000 and 20,000 MS/MS spectra to identify.

Appendix C has been removed for copyright or proprietary reasons. It contains data related to chapter 2 which also has been removed.

APPENDIX D

GROWTH RESPONSE OF *E. COLI* O157:H7 SAKAI TO TEMPERATURE DOWNSHIFT AS DETERMINED BY VIABLE COUNT

Parallel to turbidimetric technique, viable count method was employed to evaluate the growth response of *E. coli* to an abrupt downshift in temperature from 35°C to either 20°C, 17°C, 14°C or 10°C. Specifically, samples were taken periodically for determination of *E. coli* growth. This was followed by serially diluting samples in 0.1% peptone water (PW; LP0037, Oxoid, Australia; Appendix A.9) and plating their appropriate dilutions on brain-heart infusion (BHI) agars supplemented with 0.1% sodium pyruvate (P8574, Sigma, USA; Appendix A.6). All plates were then incubated at 37°C for 24 h before colonies were counted. As a result, shifting cells to low temperature was found to induce a lag period before resumption of growth at a slower rate (Fig. D.1). This pattern of growth response is similar to that from the turbidimetric data.

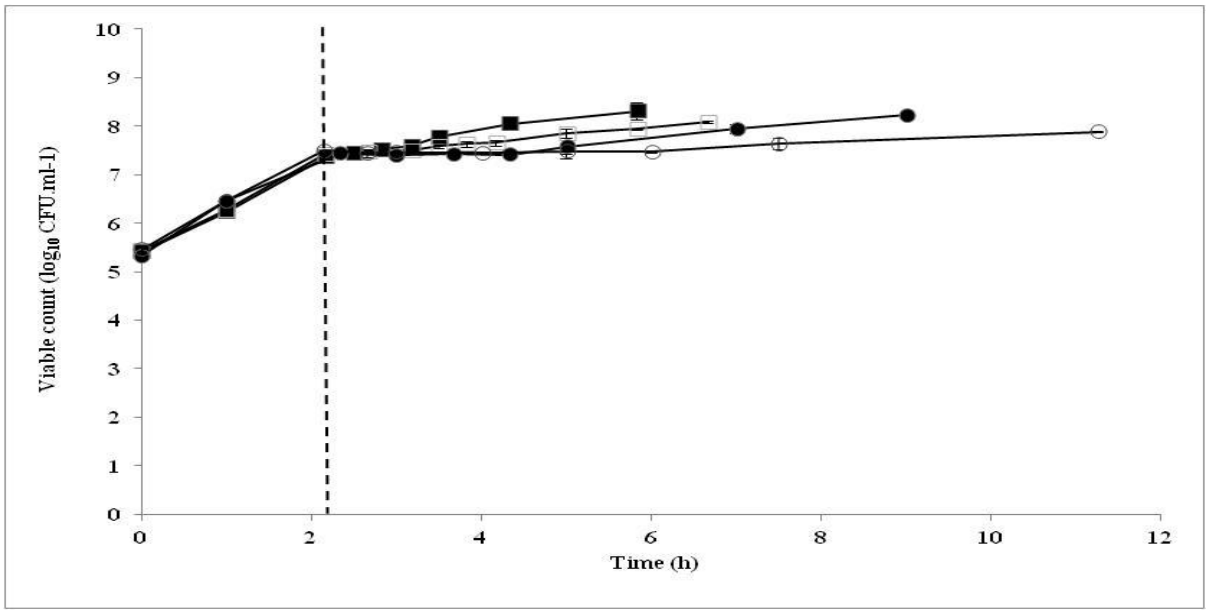

Fig. D.1. Growth response of exponential phase *E. coli* O157:H7 Sakai to a rapid downshift in temperature from 35°C to 20°C (■), 17°C (□), 14°C (●) and 10°C (○), as determined by viable count. The time at which cold shock was applied is indicated by a dotted line. Data points represent means ± standard deviations of at least two independent replicates.

APPENDIX E

TRANSCRIPTOMIC AND PROTEOMIC DATA (CHAPTER 3)

E.1 Protein identifications

A complete list of protein identifications with high confidence score in membrane and soluble fractions of *E. coli* O157:H7 Sakai during exposure to a temperature downshift is provided in Tables E.1 – E.5 (on CD-ROM).

E.2 False-positive discovery rate

The peptide false-positive discovery rate for each MudPIT run, as calculated by dividing the number of spectra matching decoy peptides with the total number of spectra (Elias et al. 2005) is summarized in Table E.6 (on CD-ROM).

E.3 Genes and proteins with significant changes in expression

A comprehensive list of differentially expressed genes and proteins with annotations as well as their comparison to previously published reports is given in Table E.7 (on CD-ROM).

E.4 Comparison of transcriptomic and proteomic profiles

Venn diagrams are provided in Fig. E.1, to describe the comparison of differentially expressed genes and proteins in response to cold stress.

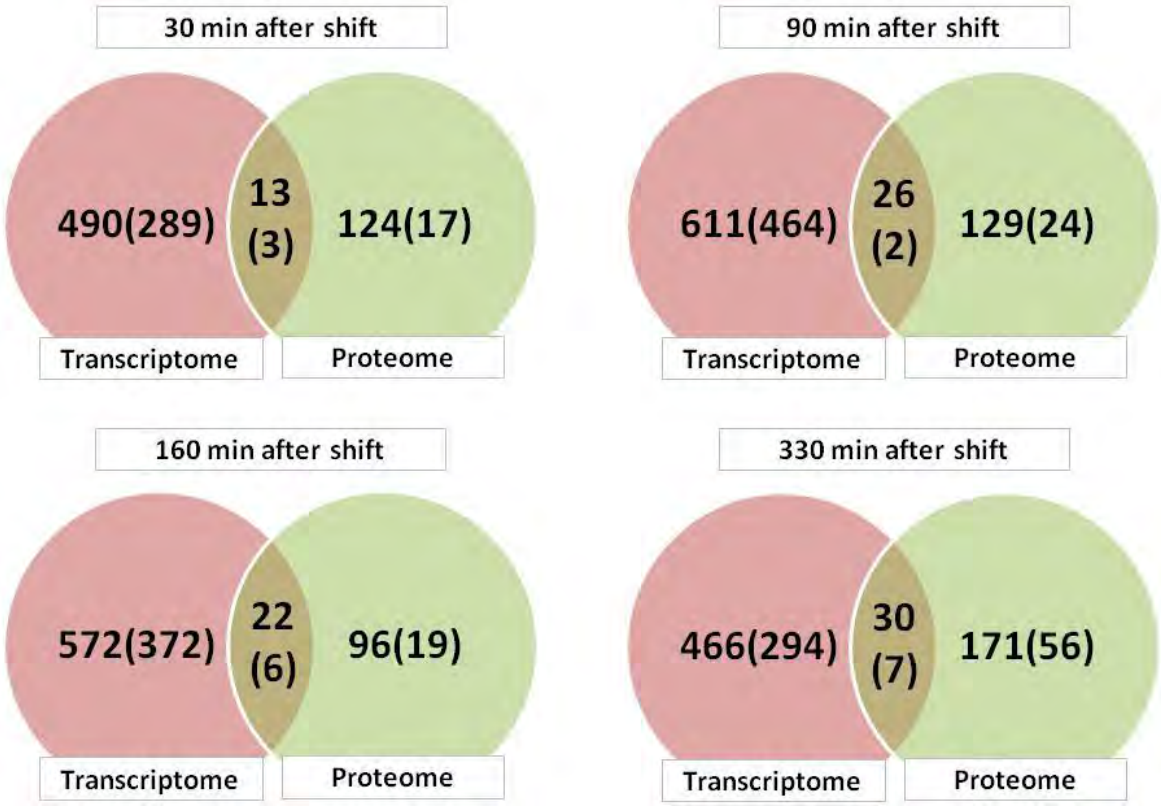

Fig. E.1. Venn diagram showing the comparison of differentially expressed genes and proteins in response to cold stress. Elements with increase expression are shown outside brackets and down-regulated elements are shown within brackets.

E.5 T-profiler analysis

Comprehensive results obtained from the T-profiler analysis of transcriptomic and proteomic data are given in Tables E.8 – E.10.

Table E.8 T-profiler analysis of transcriptomic and proteomic profiles of *E. coli* O157:H7 Sakai in response to temperature downshift from 35°C to 14°C based on JCVI CMR functional categories.

JCVI CMR functional categories[a]	30 min				90 min				160 min				330 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value[b]	*P*-value[c]	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
Amino acid biosynthesis																
Aromatic amino acid family	0.64		-1.02		**2.25**	**0.041**	0.72		1.42		0.04		1.36		0.55	
Aspartate family	1.23		-1.63		0.48		-1.27		-0.30		-0.70		-1.05		**-2.29**	**0.037**
Glutamate family	-1.74		-0.51		-0.93		-0.74		1.31		-0.93		1.04		-0.39	
Pyruvate family	-0.47		-0.51		-0.80		-0.19		**-2.12**	**0.057**	-0.99		**-3.43**	**0.006**	-1.50	
Serine family	-1.07		0.94		-0.68		0.60		-1.10		0.13		0.66		-0.61	
Histidine family	1.36		-0.29		**2.42**	**0.052**	0.30		1.60		-0.08		**3.82**	**0.009**	0.18	
Biosynthesis of cofactors, prosthetic groups, and carriers																
Biotin	1.37		ND		1.65		ND		0.38		ND		0.46		ND	
Folic acid	**-2.53**	**0.035**	**-2.20**	**0.064**	**-2.84**	**0.022**	-1.69		**-1.99**	**0.082**	**-2.18**	**0.065**	-0.68		**-2.24**	**0.060**
Heme, porphyrin, and cobalamin	-1.13		-0.50		-0.69		0.13		-1.29		0.34		**-2.17**	**0.049**	-0.07	
Menaquinone and ubiquinone	-0.55		-1.25		-1.38		-1.40		-1.01		**-1.95**	**0.079**	-1.11		-1.23	
Molybdopterin	-1.63		-2.19		-1.82		-1.20		-0.11		0.06		-0.77		-1.62	
Pantothenate and coenzyme A	ND		-1.38		ND		-1.56		ND		-2.11		ND		-2.03	
Pyridoxine	ND		-0.78		ND		-1.30		ND		-0.79		ND		-0.68	
Riboflavin, FMN, and FAD	0.64		-0.76		0.40		-0.34		-0.12		-1.26		0.38		-0.84	
Glutathione and analogs	ND[d]		ND		ND		ND		ND		ND		ND		ND	
Thiamine	0.76		-1.51		-0.36		-1.42		-0.20		-1.51		-0.08		-0.91	
Cell envelope																
Surface structures	1.19		-1.63		-0.04		-1.40		-0.76		-0.55		**-2.33**	**0.025**	-2.07	
Biosynthesis and degradation of murein sacculus and peptidoglycan	0.78		1.41		-0.04		0.41		-1.19		0.36		-0.92		-0.21	
Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	-1.22		**-1.67**	**0.090**	-1.36		**-2.35**	**0.023**	-0.67		**-1.68**	**0.098**	**3.45**	**0.001**	-1.43	

JCVI CMR functional categories	30 min				90 min				160 min				330 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
Cellular processes																
Cell division	1.18		0.38		-0.21		-0.64		-0.05		-0.84		0.09		-0.82	
Chemotaxis and motility	1.20		1.20		**-6.87**	**<0.001**	0.19		**-15.07**	**<0.001**	0.02		**-11.67**	**<0.001**	-0.99	
Detoxification	-0.87		0.42		0.02		0.27		0.61		1.00		-0.10		0.69	
DNA transformation	ND		ND		ND		ND		ND		ND		ND		ND	
Toxin production and resistance	0.80		-0.95		0.07		**-2.43**	**0.024**	-0.67		-1.32		0.39		**-1.86**	**0.075**
Pathogenesis	0.20		0.35		1.63		-0.09		0.92		-0.16		-0.57		0.06	
Adaptations to atypical conditions	**2.49**	**0.023**	**2.32**	**0.053**	**2.08**	**0.053**	**3.29**	**0.013**	**1.82**	**0.087**	**3.17**	**0.013**	**2.96**	**0.009**	1.53	
Central intermediary metabolism																
Amino sugars	ND		ND		ND		ND		ND		ND		ND		ND	
Phosphorus compounds	1.44		ND		2.13		ND		**2.36**	**0.078**	ND		0.83		ND	
Polyamine biosynthesis	ND		-0.03		ND		-0.49		ND		-0.09		ND		-1.54	
Sulfur metabolism	**-3.47**	**0.008**	0.66		**-3.29**	**0.011**	0.82		**-3.45**	**0.009**	0.26		0.38		-0.15	
Nitrogen metabolism	-1.40		ND		-1.06		ND		-1.35		-2.21		**-2.68**	**0.037**	ND	
DNA metabolism																
DNA replication, recombination, and repair	-0.12		**-1.92**	**0.060**	0.04		-1.46		-0.30		**-2.55**	**0.013**	0.17		**-2.18**	**0.033**
Restriction/modification	ND		0.20		ND		0.58		ND		0.31		ND		-0.05	
Degradation of DNA	ND		0.16		ND		-0.14		ND		ND		ND		0.75	
Energy metabolism																
Aerobic	-1.30		**1.93**	**0.077**	**-2.86**	**0.013**	**1.98**	**0.071**	**-2.58**	**0.022**	**1.81**	**0.095**	-0.14		**2.23**	**0.045**
Amino acids and amines	**-1.77**	**0.089**	-0.74		-1.63		-0.86		-0.15		0.74		-0.01		-0.06	
Anaerobic	0.81		-0.57		0.97		-0.29		0.92		-0.23		0.57		0.07	
ATP-proton motive force interconversion	0.15		1.87		-1.48		1.46		**-2.50**	**0.067**	1.37		**-2.17**	**0.096**	1.00	
Electron transport	**-2.80**	**0.006**	-0.32		**-2.47**	**0.015**	0.19		**-2.35**	**0.021**	1.52		-1.21		0.75	
Entner-Doudoroff	ND		ND		ND		ND		ND		ND		ND		ND	

JCVI CMR functional categories	30 min				90 min				160 min				330 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
Fermentation	-1.22		-0.31		0.10		1.09		0.38		0.82		-0.02		1.29	
Glycolysis/gluconeogenesis	**-2.28**	**0.032**	-0.68		-0.19		0.18		0.06		0.82		0.56		-0.57	
Pentose phosphate pathway	**-1.84**	**0.099**	-0.09		-0.81		-0.33		0.93		0.99		0.15		0.54	
Pyruvate dehydrogenase	-0.14		-0.01		0.02		0.76		-2.00		0.46		**-3.45**	**0.041**	-0.99	
Sugars	**-2.46**	**0.018**	-0.65		-1.06		-1.73		0.63		-1.34		-0.42		-0.97	
TCA cycle	**1.91**	**0.072**	-0.60		**3.06**	**0.007**	0.52		**2.28**	**0.035**	**3.36**	**0.003**	**2.80**	**0.012**	**2.75**	**0.014**
Methanogenesis	ND		ND		ND		ND		ND		ND		ND		ND	
Biosynthesis and degradation of polysaccharides	-1.01		-0.28		0.61		-0.60		1.56		0.69		0.16		0.68	
Photosynthesis	ND		ND		ND		ND		ND		ND		ND		ND	
Fatty acids and phospholipids metabolism																
Biosynthesis	-0.67		-0.72		-0.75		**-1.88**	**0.073**	-1.59		-0.96		**-2.19**	**0.037**	**-1.75**	**0.093**
Degradation	-0.13		0.06		0.52		0.35		0.76		0.02		-0.20		-0.32	
Mobile and extrachromosomal element functions																
Prophage functions	-1.04		-0.90		0.56		-0.46		0.03		-0.53		-0.74		-0.04	
Transposon functions	1.66		ND		**1.78**	**0.096**	ND		1.36		ND		1.37		ND	
Protein fate																
Protein and peptide secretion and trafficking	0.31		1.38		0.17		0.71		0.53		0.64		-0.47		0.84	
Protein modification and repair	0.92		-0.72		0.87		0.65		0.55		-1.63		0.67		-0.53	
Protein folding and stabilization	**-2.76**	**0.012**	-0.64		**-3.45**	**0.003**	0.31		-1.29		0.20		-0.63		-1.04	
Degradation of proteins, peptides, and glycopeptides	**-1.85**	**0.074**	0.86		-0.62		1.19		-0.13		1.04		-0.77		1.26	
Protein synthesis																
tRNA aminoacylation	**-1.85**	**0.079**	0.45		**-2.02**	**0.057**	0.25		**-2.80**	**0.011**	-0.58		**-2.29**	**0.033**	-1.14	
Ribosomal proteins: synthesis and modification	**-2.08**	**0.050**	0.74		-1.43		0.29		-1.04		-0.48		**-1.94**	**0.066**	-0.05	
tRNA and rRNA base modification	-1.18		-1.46		**-1.75**	**0.096**	**-2.01**	**0.055**	**-1.93**	**0.069**	**-2.01**	**0.055**	-0.01		**-2.88**	**0.008**
Translation factors	-0.79		-0.82		-0.89		-0.99		-0.32		-0.41		0.71		-0.37	

JCVI CMR functional categories	30 min				90 min				160 min				330 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
Purines, pyrimidines, nucleosides, and nucleotides																
2'-Deoxyribonucleotide metabolism	**-3.48**	**0.007**	ND		**-3.69**	**0.005**	ND		**-3.48**	**0.007**	ND		**-3.94**	**0.003**	ND	
Nucleotide and nucleoside interconversions	ND		-0.35		ND		-0.18		ND		-0.81		ND		-0.88	
Purine ribonucleotide biosynthesis	**-3.67**	**0.006**	-0.88		**-3.69**	**0.006**	**-1.80**	**0.093**	**-2.28**	**0.052**	-1.32		-0.24		-1.60	
Pyrimidine ribonucleotide biosynthesis	-1.54		-0.76		-1.48		-1.12		-0.71		-0.79		0.08		-0.82	
Salvage of nucleoside and nucleotide	**-4.78**	**<0.001**	0.32		**-4.03**	**0.001**	-0.28		**-2.27**	**0.035**	-0.53		**-2.54**	**0.020**	-1.27	
Sugar-nucleotide biosynthesis and conversions	-0.67		0.03		0.60		0.40		0.74		-0.37		1.43		0.01	
Regulatory functions																
DNA interactions	**2.48**	**0.021**	0.00		1.50		1.33		0.94		-0.71		1.67		0.84	
Protein interactions	ND		ND		ND		ND		ND		ND		ND		ND	
Transcription																
Degradation of RNA	-1.50		-0.59		-1.39		-0.88		-0.75		-0.45		-0.93		-0.95	
DNA-dependent RNA polymerase	ND		2.05		ND		2.00		ND		**2.21**	**0.078**	ND		1.45	
Transcription factors	**2.01**	**0.063**	0.65		1.24		1.30		0.24		0.99		1.02		1.39	
RNA processing	0.64		-1.11		1.21		-0.45		0.46		-1.89		0.95		-1.09	
Transport and binding proteins																
Amino acids, peptides and amines	0.48		**3.14**	**0.004**	0.43		**3.77**	**0.001**	0.96		**4.04**	**<0.001**	0.02		**4.76**	**0.001**
Anions	**-4.19**	**0.001**	1.59		**-3.57**	**0.003**	1.22		**-3.95**	**0.001**	0.38		-0.58		0.74	
Carbohydrates, organic alcohols, and acids	**-2.09**	**0.040**	1.23		**-1.90**	**0.062**	1.00		-1.62		0.97		**-2.28**	**0.025**	1.66	
Cations and iron carrying compounds	-0.73		**4.52**	**<0.001**	**-4.32**	**<0.001**	**3.18**	**0.003**	**-4.84**	**<0.001**	**3.23**	**0.003**	**-5.02**	**<0.001**	**3.41**	**0.002**
Nucleosides, purines and pyrimidines	**-2.33**	**0.067**	ND		**-2.63**	**0.047**	ND		-1.80		ND		0.24		ND	

[a] JCVI CMR role categories were obtained from http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi.

[b] *T*-values were determined from $\log_2$ expression data (see Section 3.2.10). Boldface type indicates significant *T*-values.

[c] *P*-values <0.1 only are shown.

[d] ND, not determined.

Table E.9 T-profiler analysis of transcriptomic and proteomic profiles of *E. coli* O157:H7 Sakai in response to temperature downshift from 35°C to 14°C based on Ecocyc and KEGG functional categories.

Ecocyc and KEGG functional categories[a]	30 min				90 min				160 min				330 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value[b]	*P*-value[c]	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
Carbohydrate catabolism and energy generation																
Ecocyc: Glycolysis	**-3.69**	**<0.001**	-0.62		**-2.42**	**0.034**	-0.18		-1.07		0.15		-0.29		-0.68	
Ecocyc: Gluconeogenesis	**-2.75**	**0.020**	-0.54		-1.56		0.34		-0.57		0.71		-0.15		-0.58	
Ecocyc: Pentose phosphate pathway	**-2.49**	**0.055**	-0.43		-1.42		-0.91		0.86		0.75		0.12		-0.13	
Ecocyc: TCA cycle and glyoxylate bypass	**1.92**	**0.084**	0.26		**3.16**	**0.010**	0.52		**2.87**	**0.017**	**3.07**	**0.009**	**3.34**	**0.01**	**2.91**	**0.013**
Ecocyc: Mix acid fermentation	**-1.81**	**0.094**	0.38		-0.11		0.63		0.14		**2.16**	**0.045**	-0.60		0.76	
KEGG: Oxidative phosphorylation	**-3.26**	**<0.001**	**3.14**	**<0.001**	**-4.74**	**<0.001**	**3.01**	**0.006**	**-4.85**	**<0.001**	**3.11**	**<0.001**	-1.53		**3.23**	**<0.001**
Transcription and translation																
Ecocyc: RNA polymerase	ND[d]		1.08		ND		0.88		ND		0.86		ND		0.72	
Ecocyc: Sigma factor	1.92		ND		1.19		ND		0.09		ND		0.10		ND	
KEGG: Ribosomal proteins	-0.45		1.27		-0.30		0.80		0.10		0.14		0.58		0.57	
Cellular process																
KEGG: Bacterial chemotaxis and motility	0.60		0.88		**-6.60**	**<0.001**	0.73		**-14.07**	**0.000**	0.57		**-10.59**	**<0.001**	-0.40	
Fatty acids and lipids metabolism																
Ecocyc: Superpathway of lipopolysaccharide biosynthesis	-0.51		**-2.71**	**0.015**	-1.47		**-2.90**	**0.010**	-1.79		**-2.13**	**0.049**	-0.28		**-1.93**	**0.071**
Ecocyc: Superpathway of fatty acid biosynthesis I (E. coli)	ND		0.08		ND		-0.97		ND		-0.50		ND		-0.52	
Ecocyc: Superpathway of unsaturated fatty acids biosynthesis	ND		0.81		ND		-0.17		ND		0.64		ND		0.08	
Ecocyc: Phospholipid biosynthesis I	-0.47		1.12		-0.33		1.42		-0.41		1.31		-0.37		0.84	
Ecocyc: Fatty acid β-oxidation I	-0.01		ND		0.67		ND		0.44		ND		-0.03		ND	

Ecocyc and KEGG functional categories	30 min				90 min				160 min				330 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
Cell structures biosynthesis																
Ecocyc: Peptidoglycan biosynthesis I (meso-diaminopimelate containing)	0.61		0.19		-0.87		-0.10		-1.88		-0.61		-1.65		-0.59	
Ecocyc: Superpathway of KDO2-lipid A biosynthesis	ND		**-3.13**	**0.012**	ND		**-3.28**	**0.010**	ND		**-1.84**	**0.099**	ND		-1.67	
Ecocyc: Enterobacterial common antigen biosynthesis	1.07		-1.66		0.18		-1.86		-0.54		-1.78		0.10		-1.50	

[a] Ecocyc functional categories were obtained from http://www.ecocyc.org and KEGG functional categories were obtained from http://www.genome.jp/kegg.

[b] *T*-values were determined from $\log_2$ expression data (see Section 3.2.10). Boldface type indicates significant *T*-values.

[c] *P*-values <0.1 only are shown.

[d] ND, not determined.

Table E.10 T-profiler analysis of transcriptomic and proteomic profiles of *E. coli* O157:H7 Sakai in response to temperature downshift from 35°C to 14°C based on major regulons.

Major regulons[a]	30 min				90 min				160 min				330 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value[b]	*P*-value[c]	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
CpxRA regulon	**2.01**	**0.062**	1.06		**2.43**	**0.027**	1.05		**2.08**	**0.054**	1.73		**1.94**	**0.070**	0.91	
Rcs regulon	**-1.73**	**0.095**	ND[d]		-0.11		ND		**2.65**	**0.013**	1.84		**7.62**	**<0.001**	0.43	
RpoE regulon	**1.95**	**0.056**	1.09		1.31		1.23		-0.20		0.50		0.11		0.19	
RpoH regulon	**-3.63**	**<0.001**	**-1.66**	**0.093**	**-3.56**	**<0.001**	-0.61		-0.25		-1.03		-0.64		-1.64	
RpoS regulon	-0.42		0.94		**1.84**	**0.069**	**1.92**	**0.066**	**10.50**	**<0.001**	**4.47**	**<0.001**	**8.27**	**<0.001**	**4.22**	**<0.001**

[a] Genes/proteins previously shwon to be positively controlled by CpxRA, Rcs, RpoE, RpoH, and RpoS regulons (Section 3.2.10).

[b] *T*-values were determined from $\log_2$ expression data (see Section 3.2.10). Boldface type indicates significant *T*-values.

[c] *P*-values <0.1 only are shown.

[d] ND, not determined.

APPENDIX F

TRANSCRIPTOMIC AND PROTEOMIC DATA (CHAPTER 4)

F.1 Protein identifications

A complete list of protein identifications with high confidence score in membrane and soluble fractions of *E. coli* O157:H7 Sakai during exposure to a downshift in water activity is provided in Tables F.1 – F.5 (on CD-ROM).

F.2 False-positive discovery rate

The peptide false-positive discovery rate for each MudPIT run, as calculated by dividing the number of spectra matching decoy peptides with the total number of spectra (Elias et al. 2005) is summarized in Table F.6 (on CD-ROM).

F.3 Genes and proteins with significant changes in expression

A comprehensive list of differentially expressed genes and proteins with annotations as well as their comparison to previously published reports is given in Table F.7 (on CD-ROM).

F.4 Comparison of transcriptomic and proteomic profiles

Venn diagrams are provided in Fig. F.1, to describe the comparison of differentially expressed genes and proteins in response to osmotic stress.

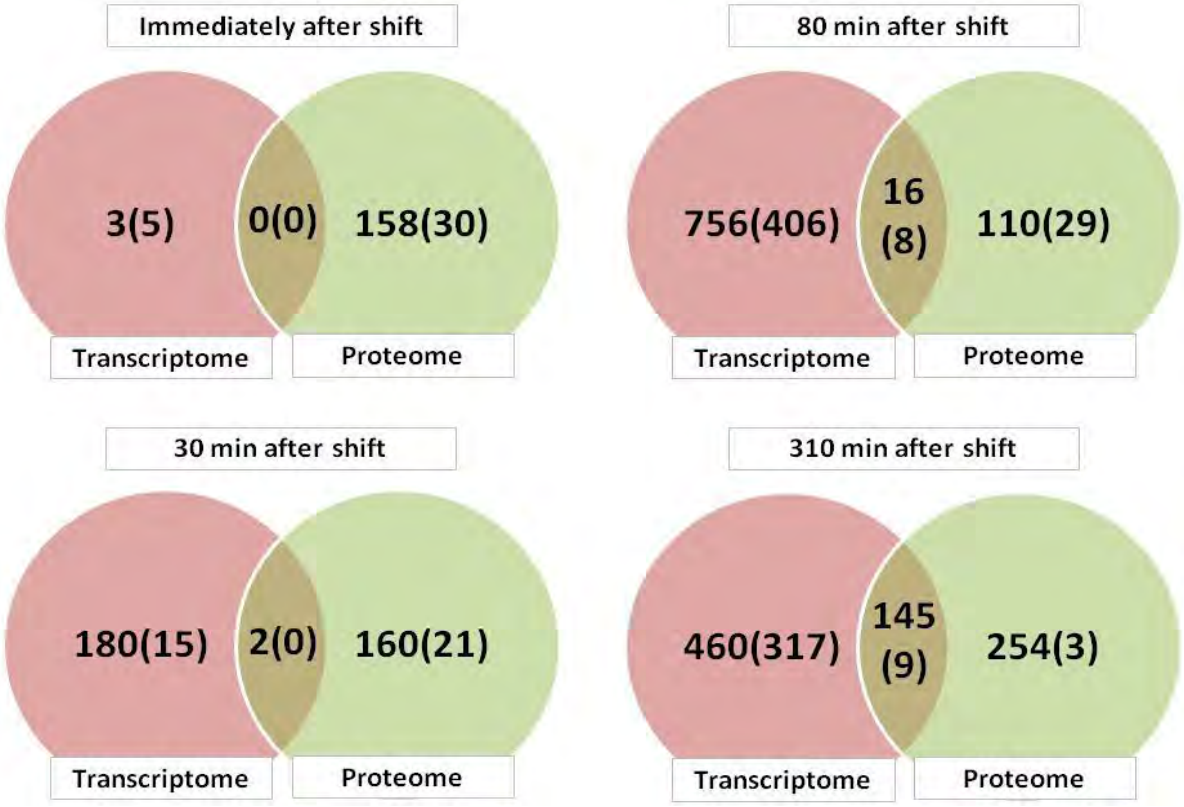

Fig. F.1. Venn diagram showing the comparison of differentially expressed genes and proteins in response to osmotic stress. Elements with increase expression are shown outside brackets and down-regulated elements are shown within brackets.

F.5 T-profiler analysis

Comprehensive results obtained from the T-profiler analysis of transcriptomic and proteomic data are given in Tables F.8 – F.10.

Table F.8 T-profiler analysis of transcriptomic and proteomic profiles of *E. coli* O157:H7 Sakai in response to hyperosmotic shift from a_w 0.993 to a_w 0.967 based on JCVI CMR functional categories.

JCVI CMR functional categories[a]	Immediately after shift				30 min				80 min				310 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value[b]	*P*-value[c]	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
Amino acid biosynthesis																
Aromatic amino acid family	-1.18		-1.73		-0.95		-1.59		-1.07		-1.60		-0.62		0.19	
Aspartate family	0.87		-0.50		0.77		-0.21		0.15		-0.62		0.92		0.61	
Glutamate family	-0.78		-0.54		-1.11		-0.34		-1.07		-0.73		-0.95		-0.72	
Pyruvate family	1.68		-0.67		0.58		-1.12		0.84		-1.15		**2.62**	**0.024**	**3.22**	**0.015**
Serine family	-0.16		0.67		-0.66		0.09		-0.89		0.38		-0.85		0.85	
Histidine family	0.15		1.09		0.28		0.88		**-2.31**	**0.061**	0.01		-1.20		0.24	
Biosynthesis of cofactors, prosthetic groups, and carriers																
Biotin	-1.15		ND		-0.42		ND		-0.95		ND		-0.80		ND	
Folic acid	0.90		-0.88		-1.46		-1.24		**-2.77**	**0.024**	-1.38		-1.32		-1.70	
Heme, porphyrin, and cobalamin	-0.97		0.02		-1.35		0.81		-0.95		-0.05		-1.45		0.61	
Menaquinone and ubiquinone	-1.16		-0.70		-0.02		-0.42		-1.11		**-2.25**	**0.046**	-1.18		-1.35	
Molybdopterin	-0.82		-0.50		0.69		-0.14		-1.49		-1.35		-0.29		-0.41	
Pantothenate and coenzyme A	ND[d]		-0.54		ND		-0.35		ND		0.25		ND		-0.34	
Pyridoxine	ND		-0.70		ND		-0.61		ND		-0.45		ND		-0.66	
Riboflavin, FMN, and FAD	0.71		-1.28		-1.12		**-2.38**	**0.098**	-1.37		-1.83		-0.68		-0.92	
Glutathione and analogs	ND		ND		ND		ND		ND		ND		ND		1.13	
Thiamine	-0.44		-0.80		-0.61		-0.41		-1.51		-1.08		-0.91		-0.96	
Cell envelope																
Surface structures	-1.33		**-3.21**	**0.033**	**3.15**	**0.003**	**-2.93**	**0.043**	**2.50**	**0.017**	**-2.58**	**0.062**	-0.25		-1.00	
Biosynthesis and degradation of murein sacculus and peptidoglycan	-0.43		1.43		0.29		1.16		-1.66		0.61		-0.50		-1.33	
Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	0.94		**1.72**	**0.092**	1.34		**2.21**	**0.031**	**2.36**	**0.022**	0.36		**4.19**	**<0.001**	1.57	

JCVI CMR functional categories	Immediately after shift				30 min				80 min				310 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
Cellular processes																
Cell division	0.67		-0.84		0.23		-0.48		-0.20		-0.83		0.76		0.36	
Chemotaxis and motility	**2.93**	**0.005**	**1.78**	**0.088**	0.34		0.24		**-6.74**	**<0.001**	-1.05		**-25.63**	**<0.001**	**-6.51**	**<0.001**
Detoxification	0.15		0.54		-0.84		-0.22		1.60		0.28		**2.72**	**0.017**	0.74	
DNA transformation	ND		ND		ND		ND		ND		ND		ND		ND	
Toxin production and resistance	0.61		-0.04		-0.21		-0.49		-1.09		-1.57		-0.27		-1.58	
Pathogenesis	-0.85		-1.05		1.36		-1.10		**2.55**	**0.034**	-0.66		0.95		-1.05	
Adaptations to atypical conditions	**2.70**	**0.015**	-1.05		1.03		-1.09		**2.16**	**0.045**	1.72		**1.77**	**0.095**	1.64	
Central intermediary metabolism																
Amino sugars	ND		ND		ND		ND		ND		ND		ND		ND	
Phosphorus compounds	-1.19		ND		0.49		ND		0.65		ND		1.49		ND	
Polyamine biosynthesis	ND		0.45		ND		0.24		ND		0.42		ND		-0.14	
Sulfur metabolism	**-2.23**	**0.057**	-0.25		-0.79		-0.74		-1.01		-0.18		0.38		0.68	
Nitrogen metabolism	-1.07		ND		-0.96		0.13		**-2.30**	**0.061**	0.74		-1.69		-0.30	
DNA metabolism																
DNA replication, recombination, and repair	**-1.69**	**0.094**	**-1.83**	**0.073**	-0.79		-1.62		-1.42		-1.09		-1.07		-1.10	
Restriction/modification	ND		-0.59		ND		-0.42		ND		-0.33		ND		-1.61	
Degradation of DNA	ND		ND		ND		ND		ND		ND		ND		-0.22	
Energy metabolism																
Aerobic	0.31		1.06		0.29		1.24		**-2.11**	**0.054**	1.19		0.56		0.39	
Amino acids and amines	-0.21		-0.91		-1.35		-1.41		-1.29		-1.12		1.50		1.54	
Anaerobic	**-2.44**	**0.050**	0.11		-1.27		-0.17		-0.89		-0.64		0.29		0.35	
ATP-proton motive force interconversion	1.25		0.80		0.58		1.34		**-2.13**	**0.100**	1.30		**-2.99**	**0.040**	-0.63	
Electron transport	0.89		-0.61		-1.64		-0.02		**-1.98**	**0.051**	-0.25		0.66		1.39	
Entner-Doudoroff	ND		ND		ND		ND		ND		ND		ND		ND	

JCVI CMR functional categories	Immediately after shift				30 min				80 min				310 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
Fermentation	**1.84**	**0.080**	-1.28		-0.58		-0.87		0.23		-0.65		1.52		1.69	
Glycolysis/gluconeogenesis	1.62		-0.26		-0.91		-0.55		0.28		0.10		**2.51**	**0.019**	**1.77**	**0.088**
Pentose phosphate pathway	0.62		-1.07		-0.44		0.34		0.44		-0.39		1.44		0.86	
Pyruvate dehydrogenase	-1.37		-0.13		-0.53		-0.29		0.36		0.22		-0.01		ND	
Sugars	1.01		-1.71		-1.49		-1.42		-0.15		**-2.20**	**0.044**	**2.35**	**0.023**	1.22	
TCA cycle	1.62		-0.86		0.36		-0.26		**2.15**	**0.045**	0.25		**2.60**	**0.018**	**3.50**	**0.002**
Methanogenesis	ND		ND		ND		ND		ND		ND		ND		ND	
Biosynthesis and degradation of polysaccharides	0.40		0.15		-1.32		0.21		-0.06		-0.72		0.80		0.06	
Photosynthesis	ND		ND		ND		ND		ND		ND		ND		ND	
Fatty acid and phospholipid metabolism																
Biosynthesis	-1.61		0.59		**-2.09**	**0.046**	0.57		**-2.55**	**0.016**	0.61		**-2.01**	**0.054**	**-2.10**	**0.046**
Degradation	0.27		0.72		-0.79		0.73		-1.19		0.59		-0.69		-0.55	
Mobile and extrachromosomal element functions																
Prophage functions	0.85		0.74		0.02		0.79		1.08		0.83		-0.09		0.76	
Transposon functions	-1.15		ND		**2.36**	**0.033**	ND		0.42		ND		0.22		ND	
Protein fate																
Protein and peptide secretion and trafficking	**-1.89**	**0.075**	-0.18		1.18		0.07		0.51		0.17		1.62		-0.28	
Protein modification and repair	0.33		0.02		-0.58		-0.17		-0.13		-1.34		-1.30		-0.54	
Protein folding and stabilization	1.14		-0.99		0.29		-1.02		0.08		-0.50		-0.87		-0.59	
Degradation of proteins, peptides, and glycopeptides	-1.54		-0.42		**-1.95**	**0.060**	-0.09		-0.66		0.39		-0.36		-0.36	
Protein synthesis																
tRNA aminoacylation	1.38		1.07		0.37		0.62		**-2.15**	**0.044**	0.57		-1.54		**-1.82**	**0.081**
Ribosomal proteins: synthesis and modification	-0.13		-1.51		-1.31		-1.09		**-1.82**	**0.084**	0.17		-0.62		**-5.43**	**<0.001**
tRNA and rRNA base modification	0.87		-0.96		-0.86		-0.11		**-2.48**	**0.023**	-1.59		**-3.84**	**0.001**	**-3.48**	**0.002**
Translation factors	0.40		-1.10		-0.26		-0.91		-0.99		-0.82		-1.03		**-1.86**	**0.088**

JCVI CMR functional categories	Immediately after shift				30 min				80 min				310 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
Purines, pyrimidines, nucleosides, and nucleotides																
2'-Deoxyribonucleotide metabolism	-0.13		1.22		-0.81		0.56		0.62		1.30		0.22		1.32	
Nucleotide and nucleoside interconversions	ND		-0.76		ND		-0.36		ND		-0.38		ND		-1.19	
Purine ribonucleotide biosynthesis	-0.21		-1.49		-0.61		**-1.96**	**0.070**	**-2.30**	**0.050**	-1.49		-0.59		-0.56	
Pyrimidine ribonucleotide biosynthesis	0.86		-0.13		-0.30		-0.06		**-2.97**	**0.041**	0.07		-2.01		-1.80	
Salvage of nucleoside and nucleotide	0.62		0.26		**-1.89**	**0.075**	-1.73		-1.72		-0.47		**-2.00**	**0.061**	-1.18	
Sugar-nucleotide biosynthesis and conversions	-1.58		-0.92		0.51		-1.00		1.12		-1.49		2.07		2.04	
Regulatory functions																
DNA interactions	0.14		-0.61		-1.44		-0.42		-0.69		-2.02		-0.21		-0.06	
Protein interactions	ND		ND		ND		ND		ND		ND		ND		ND	
Transcription																
Degradation of RNA	0.33		-0.66		-1.04		-0.48		-0.85		-0.86		-0.68		-0.68	
DNA-dependent RNA polymerase	ND		1.45		ND		1.56		ND		**2.87**	**0.035**	ND		0.81	
Transcription factors	0.25		1.23		0.27		1.23		-0.24		**2.23**	**0.047**	-1.29		-0.66	
RNA processing	1.03		-0.03		-0.33		0.26		-0.32		0.73		-0.87		**-2.50**	**0.041**
Transport and binding proteins																
Amino acids, peptides and amines	0.57		**3.31**	**0.002**	-0.71		**3.72**	**0.001**	-0.49		**3.60**	**0.001**	-0.98		**2.30**	**0.026**
Anions	0.13		1.00		**-1.99**	**0.064**	1.43		**-2.32**	**0.034**	1.38		-1.52		0.66	
Carbohydrates, organic alcohols, and acids	0.26		0.00		-0.03		-0.06		0.23		0.05		**1.67**	**0.098**	-0.02	
Cations and iron carrying compounds	**2.71**	**0.009**	**4.67**	**<0.001**	1.63		**5.10**	**<0.001**	1.40		**4.26**	**<0.001**	**-3.03**	**0.004**	**1.71**	**0.095**
Nucleosides, purines and pyrimidines	-0.37		ND		-1.06		ND		**-2.34**	**0.067**	ND		-1.17		ND	

[a] JCVI CMR role categories were obtained from http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi.

[b] *T*-values were determined from $\log_2$ expression data (see Section 4.2.11). Boldface type indicates significant *T*-values.

[c] *P*-values <0.1 only are shown.

[d] ND, not determined.

Table F.9 T-profiler analysis of transcriptomic and proteomic profiles of *E. coli* O157:H7 Sakai in response to hyperosmotic shift from a_w 0.993 to a_w 0.967 based on Ecocyc and KEGG functional categories.

Ecocyc and KEGG functional categories[a]	Immediately after shift				30 min				80 min				310 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value[b]	*P*-value[c]	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
Carbohydrate catabolism and energy generation																
Ecocyc: Glycolysis	0.79		-0.37		-1.44		-0.18		-0.82		-0.04		1.77		0.36	
Ecocyc: Gluconeogenesis	0.46		-0.63		-1.13		-0.43		-0.51		-0.16		1.76		0.40	
Ecocyc: Pentose phosphate pathway	0.82		-1.01		-0.29		0.30		0.93		-0.32		1.80		1.10	
Ecocyc: TCA cycle and glyoxylate bypass	-0.53		-0.11		-0.30		0.55		**2.80**	**0.019**	0.29		**2.93**	**0.015**	**2.80**	**0.013**
Ecocyc: Mix acid fermentation	0.59		-0.66		0.30		-0.78		0.43		-1.20		1.46		0.83	
KEGG: Oxidative phosphorylation	0.15		**2.17**	**0.041**	-0.34		**2.59**	**0.016**	**-2.43**	**0.024**	**2.47**	**0.021**	0.16		0.87	
Transcription and translation																
Ecocyc: RNA polymerase	ND[d]		0.69		ND		0.69		ND		1.04		ND		-0.38	
Ecocyc: Sigma factor	0.72		ND		**2.54**	**0.064**	ND		1.45		2.32		-0.95		0.14	
KEGG: Ribosomal proteins	0.57		-1.30		1.43		-0.91		-0.31		0.49		0.56		**-4.39**	**<0.001**
Cellular process																
KEGG: Bacterial chemotaxis and motility	**2.66**	**0.011**	1.07		0.16		-0.63		**-6.13**	**<0.001**	-1.60		**-24.29**	**<0.001**	**-5.73**	**<0.001**
Fatty acids and lipids metabolism																
Ecocyc: Superpathway of lipopolysaccharide biosynthesis	0.67		0.13		-0.82		0.65		**-2.44**	**0.050**	0.03		**-2.21**	**0.070**	-1.19	
Ecocyc: Superpathway of fatty acid biosynthesis I (E. coli)	ND		0.89		ND		0.83		ND		0.65		ND		-0.82	
Ecocyc: Superpathway of unsaturated fatty acids biosynthesis	ND		1.22		ND		1.27		ND		1.14		ND		-0.32	
Ecocyc: Phospholipid biosynthesis I	-1.07		**2.22**	**0.090**	-1.71		**2.21**	**0.092**	-1.44		1.59		-0.81		0.87	
Ecocyc: Fatty acid β-oxidation I	0.67		ND		-0.20		ND		0.35		ND		1.24		ND	

Ecocyc and KEGG functional categories	Immediately after shift				30 min				80 min				310 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
Cell structures biosynthesis																
Ecocyc: Peptidoglycan biosynthesis I (meso-diaminopimelate containing)	0.74		0.33		0.22		-0.29		**-1.93**	**0.095**	-0.31		-1.02		-0.99	
Ecocyc: Superpathway of KDO2-lipid A biosynthesis	ND		-0.73		ND		-0.54		ND		-0.28		ND		-1.36	
Ecocyc: Enterobacterial common antigen biosynthesis	-0.97		0.22		-0.45		0.07		-1.73		-0.91		-1.36		-1.79	

[a] Ecocyc functional categories were obtained from http://www.ecocyc.org and KEGG functional categories were obtained from http://www.genome.jp/kegg.

[b] *T*-values were determined from $\log_2$ expression data (see Section 4.2.11). Boldface type indicates significant *T*-values.

[c] *P*-values <0.1 only are shown.

[d] ND, not determined.

Table F.10 T-profiler analysis of transcriptomic and proteomic profiles of *E. coli* O157:H7 Sakai in response to hyperosmotic shift from a_w 0.993 to a_w 0.967 based on major regulons.

Major regulons[a]	Immediately after shift				30 min				80 min				310 min			
	Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome		Transcriptome		Proteome	
	T-value[b]	*P*-value[c]	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value	*T*-value	*P*-value
CpxRA regulon	-0.05		-0.20		-1.34		0.10		0.05		0.08		-0.40		-0.48	
Rcs regulon	0.29		ND[d]		**7.16**	**<0.001**	ND		**13.57**	**<0.001**	1.66		**16.54**	**<0.001**	**10.83**	**<0.001**
RpoE regulon	1.14		-0.06		-0.20		0.66		-0.02		0.61		-0.58		-0.65	
RpoH regulon	0.81		-0.28		-0.80		-0.47		1.19		-0.64		**-2.23**	**0.030**	0.50	
RpoS regulon	**2.52**	**0.013**	0.23		**2.80**	**0.006**	**2.16**	**0.041**	**14.06**	**<0.001**	**4.48**	**<0.001**	**15.76**	**<0.001**	**13.13**	**<0.001**

[a] Genes/proteins previously shwon to be positively controlled by CpxRA, Rcs, RpoE, RpoH, and RpoS regulons (Section 4.2.11).

[b] *T*-values were determined from $\log_2$ expression data (see Section 4.2.11). Boldface type indicates significant *T*-values.

[c] *P*-values <0.1 only are shown.

[d] ND, not determined.

APPENDIX G

PROTEOMIC DATA (CHAPTER 5)

G.1 Protein identifications

A complete list of protein identifications with high confidence score in membrane and soluble fractions of *E. coli* O157:H7 Sakai during exposure to simultaneous sudden downshifts in temperature and water activity is provided in Tables G.1 – G.9 (on CD-ROM).

G.2 False-positive discovery rate

The peptide false-positive discovery rate for each MudPIT run, as calculated by dividing the number of spectra matching decoy peptides with the total number of spectra (Elias et al. 2005) is summarized in Table G.10 (on CD-ROM).

G.3 Proteins with significant changes in abundance

A comprehensive list of differentially expressed proteins with annotations as well as their comparison to previously published reports is given in Table G.11 (on CD-ROM).

G.4 T-profiler analysis

Comprehensive results obtained from the T-profiler analysis of proteomic data are given in Tables G.12 – G.14 (on CD-ROM).

G.5 Hierarchical clustering analysis of proteomic data

A high-quality version of figure is given in Fig. G.1 (on CD-ROM) to illustrate clustering analysis of proteomic data based on the T-profiler results.